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(57) Abstract

DNA sequences from the Internal Transcribed Spacer of the ribosomal RNA gene region are described for different species and strains of *Septoria, Pseudocercosporella, Fusarium* and *Mycosphaerella*. Specific primers from within these sequences are identified as being useful for the identification of the fungal isolates using PCR-based techniques.

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DETECTION OF FUNGAL PATHOGENS USING THE POLYMERASE CHAIN REACTION

FIELD OF THE INVENTION

The present invention relates to the use of species-specific primers in polymerase chain reaction assays for the detection of fungal pathogens. The use of these primers enables the detection of specific isolates of fungal pathogens and the monitoring of disease development in plant populations.

BACKGROUND OF THE INVENTION

Diseases in plants cause considerable crop loss from year to year resulting both in economic deprivation to farmers and additionally in many parts of the world to shortfalls in the nutritional provision for local populations. The widespread use of fungicides has provided considerable security against plant pathogen attack. However, despite $1 billion worth of expenditure on fungicides, worldwide crop losses amounted to approximately 10% of crop value in 1981 (James, 1981; *Seed Sci. & Technol.* 9: 679-685).

The severity of the destructive process of disease depends on the aggressiveness of the pathogen and the response of the host. One aim of most plant breeding programs is to increase the resistance of host plants to disease. Typically, different races of pathogens interact with different varieties of the same crop species differentially, and many sources of host resistance only protect against specific pathogen races. Furthermore, some pathogen races show early signs of disease symptoms, but cause little damage to the crop. Jones and Clifford (1983; Cereal Diseases, John Wiley) report that virulent forms of the pathogen are expected to emerge in the pathogen population in response to the introduction of resistance into host cultivars and that it is therefore necessary to monitor pathogen populations. In addition, there are several documented cases of the evolution of fungal strains which are

resistant to particular fungicides. As early as 1981, Fletcher and Wolfe (1981; *Proc. 1981 Brit. Crop Prot. Conf.*) contended that 24% of the powdery mildew populations from spring barley, and 53% from winter barley showed considerable variation in response to the fungicide triadimenol and that the distribution of these populations varied between varieties with the most susceptible variety also giving the highest incidence of less susceptible types. Similar variation in the sensitivity of fungi to fungicides has been documented for wheat mildew (also to triadimenol), *Botrytis* (to benomyl), *Pyrenophora* (to organomercury), *Pseudocercosporella* (to MBC-type fungicides) and *Mycosphaerella fijiensis* to triazoles to mention just a few (Jones and Clifford; Cereal Diseases, John Wiley, 1983).

Cereal species are grown world-wide and represent a major fraction of world food production. Although yield loss is caused by many pathogens, the necrotizing pathogens *Septoria* and *Pseudocercosporella* are particularly important in the major cereal growing areas of Europe and North America (Jones and Clifford; Cereal Diseases, John Wiley, 1983). In particular, the differential symptomology caused by different isolates and species of these fungi make the accurate predictive determination of potential disease loss difficult. Consequently, the availability of improved diagnostic techniques for the rapid and accurate identification of specific pathogens will be of considerable use to field pathologists.

Four *Septoria* species parasitize the small grain species. *Septoria tritici* is the causative agent of leaf blotch and is virulent on wheat but also parasitizes triticale and rye. It typically causes leaf necrosis. *Septoria nodorum* is the causative agent of glume blotch and is parasitic on wheat, triticale, rye and barley and although mainly restricted to glumes is also found on leaf blades and sheaths. *Septoria avenae* is parasitic on oats, wheat and triticale and *Septoria passerinii* is restricted to barley. *Septoria* diseases occur in all wheat growing areas at economically important levels. Different *Septoria* diseases frequently occur concurrently within fields and on individual plants, where the disease symptoms may be collectively referred to as the "Septoria complex". Typically, the most commonly found species are *S. tritici* and *S. nodorum*. According to Wiese (1977; Compendium of Wheat Diseases, Amer. Phytopath. Soc. pages 42-45), the Septoria complex presently destroys nearly 2% of the world's wheat annually, the yield loss being mainly the result of impaired grain filling. Fungicide treatments can save up to 20% in cases of severe *Septoria* infection, but it is often difficult to distinguish between the different *Septoria* species at the onset of infection and this

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makes the decision whether or not to invest in fungicide use difficult because different cultivars display differing degrees of resistance to the various *Septoria* species.

The eyespot disease of cereals is caused by the fungus *Pseudocercosporella herpotrichoides* and is restricted to the basal culm of the plant. Wheat, rye, oats and other grasses are susceptible to the eyespot disease which occurs in cool, moist climates and is prevalent in Europe, North and South America, Africa and Australia. Wheat is the most susceptible cereal species, but isolates have been identified which are also virulent on other cereals. The R-strain of the fungus, for example, has also been isolated from rye and grows more slowly on wheat than the W-strain which has been isolated from wheat. Although eyespot may kill tillers or plants outright, it more usually causes lodging and/or results in a reduction in kernel size and number. Yield losses associated with eyespot are of even greater magnitude than those associated with *Septoria tritici* and *Septoria nodorum*. Typical control measures for eyespot include treatment with growth regulators to strengthen internodes, and fungicide treatment. However, the differing susceptibility of cultivars to different strains of the fungus render the predictive efficacy of fungicide treatments difficult.

Sigatoka leaf spot of banana occurs in two forms each of which is caused by a different fungus. The economically important Black Sigatoka is caused by *Mycosphaerella fijiensis*, whereas the less economically significant Yellow Sigatoka is caused by *Mycosphaerella musicola* (Johanson and Jeger, 1993; Mycol. Res. 97: 670-674). Black Sigatoka is the major problem in banana causing severe losses of 30% and more. Due to occurrence of fungicide resistance in *Mycosphaerella fijiensis*, usage of fungicide should best be limited to prevent the further occurrence of resistance. Consequently, the availability of diagnostic tools will provide an important means of identifying the appropriate circumstances in which to utilize fungicides without unnecessarily risking the development of further resistance.

Thus, there is a real need for the development of technology which will allow the identification of specific races of pathogen fungi early in the infection process. By identifying the specific race of a pathogen before disease symptoms become evident in the crop stand, the agriculturist can assess the likely effects of further development of the pathogen in the crop variety in which it has been identified and can choose an appropriate fungicide if such application is deemed necessary.

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SUMMARY OF THE INVENTION

The present invention is drawn to methods of identification of different pathotypes of plant pathogenic fungi. The invention provides DNA sequences which show variability between different fungal pathotypes. Such DNA sequences are useful in the method of the invention as they can be used to derive primers for use in polymerase chain reaction (PCR)-based diagnostic assays. These primers generate unique fragments in PCR reactions in which the DNA template is provided by specific fungal pathotypes and can thus be used to identify the presence or absence of specific pathotypes in host plant material before the onset of disease symptoms.

This invention provides the possibility of assessing potential damage in a specific crop variety-pathogen strain relationship and of utilizing judiciously the diverse armory of fungicides which is available. Furthermore, it can be used to provide detailed information on the development and spread of specific pathogen races over extended geographical areas. The invention provides a method of detection which is especially suitable for diseases with a long latent phase such as those caused by *Septoria nodorum* or *Septoria tritici* on wheat and *Mycosphaerella fijiensis* on banana.

Kits useful in the practice of the invention are also provided. The kits find particular use in the identification of *Septoria*, *Pseudocercosporella*, *Fusarium*, and *Mycosphaerella* pathogens.

DESCRIPTION OF THE FIGURES

Figure 1 Alignment of Internal Transcribed Spacer Sequences from *Septoria tritici*, *Septoria nodorum*, *Pseudocercosporella herpotrichoides* strain W (two variants), *Pseudocercosporella herpotrichoides* strain R, *Mycosphaerella fijiensis*, and *Mycosphaerella musicola*.

Figure 2 Alignment of the Internal Transcribed Spacer Sequences from *Septoria nodorum* and *Septoria avenae* f.sp. *triticea*.

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Figure 3 Alignment of the Internal Transcribed Spacer Sequences from *Fusarium graminearum, Fusarium culmorum, Fusarium moniliforme* and *Microdochium nivale.*

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides unique DNA sequences which are useful in identifying different pathotypes of plant pathogenic fungi. Particularly the DNA sequences can be used as primers in PCR based analysis for the identification of fungal pathotypes. The DNA sequences of the invention include the Internal Transcribed Spacer (ITS) of the ribosomal RNA gene regions of particular fungal pathogens as well as primers which are derived from these regions which are capable of identifying the particular pathogen. These ITS DNA sequences from different pathotypes within a pathogen species or genus which vary between the different members of the species or genus can be used to identify those specific members.

Biomedical researchers have used PCR-based techniques for some time and with moderate success to detect pathogens in infected animal tissues. Only recently, however, has this technique been applied to detect plant pathogens. The presence of *Gaumannomyces graminis* in infected wheat has been detected using PCR of sequences specific to the pathogen mitochondrial genome (Schlesser *et al.*, 1991; *Applied and Environ. Microbiol.* 57: 553-556) and random amplified polymorphic DNA (*i.e.* RAPD) markers were able to distinguish numerous races of *Gremmeniella abietina*, the causal agent of scleroderris canker in conifers.

Ribosomal genes are suitable for use as molecular probe targets because of their high copy number. Despite the high conservation between mature rRNA sequences, the non-transcribed and transcribed spacer sequences are usually poorly conserved and are thus suitable as target sequences for the detection of recent evolutionary divergence. Fungal rRNA genes are organized in units each of which encodes three mature subunits of 18S, 5.8S, and 28S respectively. These subunits are separated by two internal transcribed spacers, ITS1 and ITS2, of around 300 bp (White *et al.*, 1990; In: PCR Protocols; Eds.: Innes *et al.*; pages 315-322). In addition, the transcriptional units are separated by non-transcribed spacer

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sequences (NTSs). The ITS and NTS sequences are particularly suitable for the detection of specific pathotypes of different fungal pathogens.

The DNA sequences of the invention are from the Internal Transcribed Spacer (ITS) of the ribosomal RNA gene region of different plant pathogens. The ITS DNA sequences from different pathotypes within a pathogen species or genus vary between the different members of the species or genus. Once having determined the ITS sequences of a pathogen, these sequences can be aligned with other ITS sequences. In this manner, primers can be derived from the ITS sequences. That is, primers can be designed based on regions within the ITS regions that contain the greatest differences in sequence among the fungal pathotypes. These sequences and primers based on these sequences can be used to identify specific pathogen members.

Particular DNA sequences of interest include ITS DNA sequences from *Septoria*, particularly, *Septoria nodorum* and *Septoria tritici*; *Mycosphaerella*, particularly *Mycosphaerella fijiensis* and *Mycosphaerella musicola*; *Pseudocercosphorella*, particularly *Pseudocercosporella herpotrichoides*, more particularly for the W-strain and the R-strain of *Pseudocercosporella herpotrichoides, Fusarium*, particularly *F. graminearum, F. culmorum, F. moniliforme* and *Microdochium nivale*. Such ITS DNA sequences as well as primers of interest are given in SEQ ID NO: 1 - 47 and SEQ ID NO.: 50-86. The sequences find use in the PCR-based identification of the pathotypes of interest.

Methods for the use of the primer sequences of the invention in PCR analysis are well known in the art. For example, see US Patent Nos. 4,683,195 and 4,683,202 as well as Schlesser *et al.* (1991) *Applied and Environ. Microbiol. 57*:553-556. See also, Nazar *et al.* (1991; *Physiol. and Molec. Plant Pathol.* 39: 1-11) which used PCR amplification to exploit differences in the ITS regions of *Verticillium albo-atrum* and *Verticillium dahliae* and therefore distinguish between the two species; and Johanson and Jeger (1993; *Mycol. Res.* 97: 670-674) who used similar techniques to distinguish the banana pathogens *Mycosphaerella fijiensis* and *Mycospharella musicola*.

The ITS DNA sequences of the invention can be cloned from fungal pathogens by methods known in the art. In general, the methods for the isolation of DNA from fungal isolates are

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known. See, Raeder & Broda (1985) *Letters in Applied Microbiology 2*:17-20; Lee *et al.* (1990) *Fungal Genetics Newsletter 35*:23-24; and Lee and Taylor (1990) In: *PCR Protocols: A Guide to Methods and Applications*, Innes *et al.* (Eds.); pages 282-287.

Alternatively, the ITS regions of interest can be determined by PCR amplification. Primers to amplify the entire ITS region were designed according to White *et al.* (1990; In: PCR Protocols; Eds.: Innes *et al.* pages 315-322) and the amplified ITS sequence was subcloned into the pCRII cloning vector. The subcloned sequence included the lefthand ITS (ITS1), the righthand ITS (ITS2) as well as the centrally located 5.8S rRNA gene. This was undertaken for *Septoria nodorum* and *Septoria tritici*, numerous *Pseudocercosporella* isolates and *Mycosphaerella fijiensis, Mycosphaerella musicola, Septoria avenae triticea, F. graminearum, F. culmorum, F. moniliforme* and *Microdochium nivale.*

The ITS sequences were determined and within each pathogen group the sequences were compared to locate divergences which might be useful to test in PCR to distinguish the different species and/or strains. The sequences of the ITS regions which were determined are shown as Sequence ID's 1 to 6, 47, and 82-86 and also in Figures 1, 2 and 3. From the identification of divergences numerous primers were synthesized and tested in PCR-amplification. Templates used for PCR-amplification testing were firstly purified pathogen DNA, and subsequently DNA isolated from infected host plant tissue. Thus it was possible to identify pairs of primers which were diagnostic *i.e.* which identified one particular pathogen species or strain but not another species or strain of the same pathogen. Preferred primer combinations are able to distinguish between the different species or strains in infected host tissue *i.e.* host tissue which has previously been infected with a specific pathogen species or strain.

This invention provides numerous primer combinations which fulfill this criterion for different *Septoria*, *Mycosphaerella,* and *Fusarium* species and different strains of *Pseudocercosporella*. The primers of the invention are designed based on sequence differences among the fungal ITS regions. A minimum of one base pair difference between sequences can permit design of a discriminatory primer. Primers designed to a specific fungal DNA's ITS region can be used in combination with a primer made to a conserved sequence region within the ribosomal DNA's coding region to amplify species-specific PCR

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fragments. In general, primers should have a theoretical melting temperature between about 60 to about 70 degree C to achieve good sensitivity and should be void of significant secondary structure and 3' overlaps between primer combinations. Primers are generally at least about 5 to about 10 nucleotide bases.

The usefulness of cloned ITS sequences for the selection of primers for diagnostic purposes is largely due to their rapid evolutionary divergence. For example, W-type and R-type isolates of the pathogen *Pseudocercosporella herpotrichoides* were found to have divergent ITS sequences from which diagnostic primers were developed. However, the rapid divergence within the ITS sequence is apparent from the observation that two different sequence variants of the W-type were identified. The sequence identity within the W-type was 99.4 %, whereas that between W and R-types was 98.6 % suggesting a closer evolutionary relationship between the two W variants than was found between the W and the R-types. This closer relationship is also apparent from their similar host pathogenicity of the two isolates with divergent ITS sequences.

In addition to developing primers from ITS-derived sequences for PCR diagnosis of fungal isolates, the invention also encompasses the identification of primers from RAPD primer libraries which can distinguish between *Septoria nodorum* and *Septoria tritici* when used in PCR. The primers screened are commercially available and were obtained from Operon Technologies Incorporated (Alameda, CA). Screening on *Septoria* genomic DNA identified two primers which were able to detect only *S. tritici* and three which were able to detect only *S. nodorum*.

The present invention lends itself readily to the preparation of "kits" containing the elements necessary to carry out the process. Such a kit may comprise a carrier being compartmentalized to receive in close confinement therein one or more container means, such as tubes or vials. One of said container means may contain unlabeled or detectably labeled DNA primers. The labeled DNA primers may be present in lyophilized form, or in an appropriate buffer as necessary. One or more container means may contain one or more enzymes or reagents to be utilized in PCR reactions. These enzymes may be present by themselves or in admixtures, in lyophilized form or in appropriate buffers.

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Finally, the kit may contain all of the additional elements necessary to carry out the technique of the invention, such as buffers, extraction reagents, enzymes, pipettes, plates, nucleic acids, nucleoside triphosphates, filter paper, gel materials, transfer materials, autoradiography supplies, and the like.

The examples below show, without limitation, typical experimental protocols which can be used in the isolation of ITS sequences, the selection of suitable primer sequences, the testing of primers for selective and diagnostic efficacy, and the use of such primers for disease and fungal isolate detection. Such examples are provided by way of illustration and not by way of limitation.

EXAMPLES

Example 1: Fungal isolates and genomic DNA extraction

Viable fungal isolates of *S. nodorum, S. tritici, S. passerini, S. glycines, Pseudocercosporella herpotrichoides, Pseudocercosporella aestiva, Mycosphaerella citri, Mycosphaerella graminicola, Mycosphaerella fijiensis* and *Mycosphaerella musicola* were obtained from the American Type Culture Collection. *Fusarium culmorum* and *Fusarium graminearum* isolates were obtained from Dr. Paul Nelson from Penn State University. An isolate of *Michrodochium nivale* (syn. *Fusarium nivale*) was received from Ciba- Basel and an isolate of *Fusarium moniliforme* was received from Dr. Loral Castor. Fungi were grown in 150 ml potato dextrose broth inoculated with mycelial fragments from PDA (Potato Dextrose Agar) cultures. Cultures were incubated on an orbital shaker at 28°C for 7-11 days. Mycelia were pelleted by centrifugation and then ground in liquid nitrogen and total genomic DNA extracted using the protocol of Lee and Taylor (1990; In: *PCR Protocols: A Guide to Methods and Applications*; Eds.: Innes *et al.*; pages 282-287).

Dr. Bruce McDonald from Texas A&M University supplied genomic DNA from ten isolates of *S. nodorum* and nine isolates of *S. tritici*. Dr. Chris Caten of Birmingham University provided six isolates of *Septoria nodorum* purified fungal DNA. Purified genomic DNA from 12 isolates of *Pseudocercosporella herpotrichoides* was obtained from Dr. Paul Nicholson of the John Innes Centre, Norwich, UK. Six of these isolates are of the W-type;

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the other six isolates are of the R-type. These isolates were typed based on pathogenicity and RFLP studies. Andrea Johanson of the Natural Resources Institute supplied genomic DNA of six isolates of *M. musicola*, six isolates of *M. fijiensis* and a single isolate of *Mycosphaerella musae*. Purified genomic DNA from *Septoria avenae* f. sp. *triticea* ATCC#26380 was supplied by Dr. Peter Ueng from the USDA at Beltsville, Maryland.

Table 1: Source of Test Isolates

Isolate	Species	Origin	Source
ATCC#24425	*S. nodorum*	Montana	ATCC[1]
XA1.1	*S. nodorum*	Texas	B. McDonald[2]
Xa5A.2	*S. nodorum*	Texas	B. McDonald
YA3.1	*S. nodorum*	Texas	B. McDonald
XD2.1	*S. nodorum*	Texas	B. McDonald
YB2.2	*S. nodorum*	Texas	B. McDonald
93HBh6a	*S. nodorum*	Oregon	B. McDonald
93A3a	*S. nodorum*	Oregon	B. McDonald
93AYa	*S. nodorum*	Oregon	B. McDonald
93HBh8a	*S. nodorum*	Oregon	B. McDonald
93C5a	*S. nodorum*	Oregon	B. McDonald
ATCC#26517	*S. tritici*	Minnesota	ATCC
BS3	*S. nodorum*	Ireland	C. Caten[3]
BS6	*S. nodorum*	Ireland	C. Caten
BS175	*S. nodorum*	England	C. Caten
BS425	*S. nodorum*	England	C. Caten
alpha'5	*S. nodorum*	France	C. Caten
m300	*S. nodorum*	England	C. Caten
TKV2a	*S. tritici*	Turkey	B. McDonald
SYK2	*S. tritici*	Syria	B. McDonald
ISZC36.2	*S. tritici*	Israel	B. McDonald
CNRC4a.1	*S. tritici*	Canada	B. McDonald
ALA1a	*S. tritici*	Algeria	B. McDonald
ETK1	*S. tritici*	Ethiopia	B. McDonald
GEB2a.1	*S. tritici*	Germany	B. McDonald
UK92D2	*S. tritici*	United Kingdom	B. McDonald
DNB1a	*S. tritici*	Denmark	B. McDonald
ATCC#38699	*S. glycines*	Illinois	ATCC
ATCC#22585	*S. passerini*	Minnesota	ATCC
ATCC#42040	*P. herpotrichoides*-wheat		ATCC
ATCC#62012	*P. aestiva*	Germany	ATCC
ATCC#60972	*P. herp. var. herp.*- barley	Germany	ATCC
W1	*P. herpotrichoides*	United Kingdom	P. Nicholson[4]
W2	*P. herpotrichoides*	United Kingdom	P. Nicholson
W3	*P. herpotrichoides*	United Kingdom	P. Nicholson
W4	*P. herpotrichoides*	United Kingdom	P. Nicholson

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W5	*P. herpotrichoides*	New Zealand	P. Nicholson
W6	*P. herpotrichoides*	Italy	P. Nicholson
R1	*P. herpotrichoides*	Belgium	P. Nicholson
R2	*P. herpotrichoides*	New Zealand	P. Nicholson
R3	*P. herpotrichoides*	Germany	P. Nicholson
R4	*P. herpotrichoides*	Sweden	P. Nicholson
R5	*P. herpotrichoides*	United Kingdom	P. Nicholson
R6	*P. herpotrichoides*	United Kingdom	P. Nicholson
ATCC#22116	*M. fijiensis*	Philippines	ATCC
ATCC#22115	*M. musicola*	Philippines	ATCC
ATCC#24046	*M. citri*	Florida	ATCC
ATCC#62714	*M. graminicola*	Montana	ATCC
PA92	*M. fijiensis*	Panama	A. Johanson[5]
PNG291	*M. fijiensis*	Papua New Guinea	A. Johanson
GH6-3	*M. fijiensis*	Ghana	A. Johanson
TG120	*M. fijiensis*	Tonga	A. Johanson
HSB4	*M. fijiensis*	Honduras	A. Johanson
RT689	*M. fijiensis*	Rarotonga (Cook Is.)	A. Johanson
CR548	*M. musicola*	Costa Rica	A. Johanson
CM61	*M. musicola*	Cameroon	A. Johanson
CU823	*M. musicola*	Cuba	A. Johanson
MQ103	*M. musicola*	Martinique	A. Johanson
CI31	*M. musicola*	Ivory Coast	A. Johanson
CB90	*M. musicola*	Colombia	A. Johanson
BD1-4	*M. musae*	Barbados	A. Johanson
ATCC#44234	*Ceratobasidium cereale*	Netherlands	ATCC
ATCC#11404	*Drechslera sorokiniana*	Minnesota	ATCC
R-5126	*F. culmorum*	Minnesota	P. Nelson[6]
R-5106	*F. culmorum*	Michigan	P. Nelson
R-5146	*F. culmorum*	Finland	P. Nelson
R-8417	*F. graminearum*	Italy	P. Nelson
R-8422	*F. graminearum*	Canada	P. Nelson
R-8546	*F. graminearum*	Bulgaria	P. Nelson
4551	*F. moniliforme*	Indiana	L. Castor[7]
92	*M. nivale*	-----	Ciba Basel[8]
ATCC#26380	*S. avenae* f.sp.*triticea*	Minnesota	P. Ueng[9]

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1 American Type Culture Collection, Rockville, Maryland USA
2 Dr. Bruce McDonald, Texas A&M University, USA
3 Dr. Chris Caten, Birmingham University, UK
4 Dr. Paul Nicholson, John Innes Centre, UK
5 Dr. Andrea Johanson, Natural Resources Institute, UK
6 Dr. Paul Nelson, Penn State University
7 Dr. Loral Castor, Ciba Seeds Research, Bloomington, Illinois
8 Ciba-Geigy Limited, Basel, Switzerland
9 Dr. Peter Ueng, USDA, Beltsville, Maryland

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Example 2: Isolation of the internal transcribed spacer (ITS) regions

The approximately 550 bp internal transcribed spacer region fragments were PCR amplified from 25 ng of genomic DNA isolated from *S. nodorum* (ATCC#24425), *S. tritici* (ATCC#26517), *Pseudocercosporella herpotrichoides* isolates R1, R2, W2 and W5, *M. fijiensis* (ATCC#22115) and *M. musicola* (ATCC#22115) using 50 pmol of primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3'; SEQ ID NO: 38) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'; SEQ ID NO:41). PCRs were performed as described in EXAMPLE 4 except that reactions were done in 100 µl and annealing was done at of 50°C. The ITS fragments were purified by isopropanol precipitation according to Maniatis et al. (1982; *Molecular Cloning*; Eds.: Maniatis *et* al.; pages 461-462). The DNA was resuspended in 50 µl dH_2O and cloned using the Invitrogen Corporation's (San Diego, CA) TA Cloning Kit (part no. K2000-01) using the pCRII cloning vector. The DNA sequences of the ITS regions were determined by the dideoxy method using the Applied Biosystems (Foster City, CA) automated sequencer model 373A with the primers ITS1 (see sequence above), ITS2 (5'-GCTGCGTTCTTCATCGATGC-3'; SEQ ID NO:39), ITS4 (see sequence above) and the M13 universal -20 (5'-GTAAAACGACGGCCAGT-3'; SEQ ID NO:48) and Reverse (5'-AACAGCTATGACCATG-3'; SEQ ID NO:49) primers. The ITS primers ITS1 (SEQ ID NO:38), ITS2 (SEQ ID NO:39), ITS3 (SEQ ID NO:40), and ITS4 (SEQ ID NO:41) used for cloning the ITS regions are detailed in White *et al.* (1990; In: PCR Protocols; Eds.: Innes *et al.* pages 315-322).

In addition, the internal transcribed spacer regions were PCR amplified from 25 ng of genomic DNA from *S. avenae* f.sp. *triticea*, *M. nivale*, *F. moniliforme* (#4551), *F. graminearum* isolates R-8417, R-8546 and R-8422 and *F. culmorum* isolates R-5126, R-5106 and R-5146. PCR products were purified using Promega's Wizard DNA Clean-up kit (Madison, WI). The DNA sequences of the ITS regions were determined as described above using the ITS1 (SEQ ID NO:38), ITS2 (SEQ ID NO:39), ITS3 (SEQ ID NO:40) and ITS4 (SEQ ID NO:41) primers. Sequencing reactions were combined with the three isolates of *F. culmorum* and *F. graminearum* to generate a consensus sequence for *F. culmorum* and *F. graminearum*.

Example 3: DNA extraction from wheat and banana leaves

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DNA was extracted from wheat leaves using a modified version of the Rapid DNA Extraction protocol from the MicroProbe Corporation's (Garden Grove, CA) IsoQuick Nucleic Acid Extraction Kit (cat# MXT-020-100). Typical yields were 5-10 µg of total DNA from 0.2 g of leaf tissue. Approximately 100 ng of total DNA were used in each PCR assay.

Modified Rapid DNA Extraction:

Before using kit for the first time, the entire contents of Reagent 2A (20 x Dye Concentrate) were added to Reagent 2 (Extraction Matrix).

(1) Approximately 0.2 g of leaf sample were added to a 1.5 ml eppendorf tube containing 50 µl sample buffer A and 50 µl #1 lysis solution. The leaf sample was ground with a Kontes pestle.

(2) Reagent 2 (Extraction Matrix) was shaken vigorously. 350 µl of reagent 2 were added to the sample lysate.

(3) 200 µl of Reagent 3 were added (Extraction Buffer) to the sample. The sample was vortexed 20 sec.

(4) Microcentrifugation at 12,000 x g for 5 min.

(5) The aqueous phase (upper layer) was transferred to a new microcentrifuge tube. This volume was typically about 200 µl.

(6) 0.1 x the volume of the aqueous phase of Reagent 4 (Sodium Acetate) to the aqueous phase sample.

(7) An equal volume of isopropanol was added to the aqueous phase sample followed by vortexing.

(8) Microcentrifugation at 12,000 x g for 10 min.

(9) The supernatant was discarded without disturbing the nucleic acid pellet. 0.5 ml of -20°C 70% ethanol was added to the pellet. The tube was vortexed to mix.

(10) Microcentrifugation at 12,000 x g for 5 min.

(11) The supernatant was discarded and the pellet was allowed to dry.

(12) The nucleic acid pellet was dissolved in 50 µl Reagent 5 (RNase-free water).

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Example 4: Polymerase chain reaction amplification

Polymerase chain reactions were performed with the GeneAmp Kit from Perkin-Elmer/Cetus (Norwalk, CT; part no. N808-0009) using 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris-HCl, pH8.3, containing 100 µM of each TTP, dATP, dCTP, and dGTP, 50 pM primer, 2.5 units of *Taq* polymerase and 25 ng of genomic DNA in a final volume of 50 µl. Reactions were run for 30 cycles of 15 s at 94°C, 15 s at 50°C, 60°C or 70°C, and 45 s at 72°C in a Perkin-Elmer/Cetus Model 9600 thermal cycler. The products were analyzed by loading 20 µl of each PCR sample on a 1.1-1.2% agarose gel and electrophoresed.

Example 5: Synthesis and Purification of Oligonucleotides

Oligonucleotides (primers) were synthesized on an Applied Biosystems 380A DNA synthesizer using B-cyanothyl-phosphoramidite chemistry.

Example 6: Selection of species-specific primers

The ITS sequences of *S. nodorum*, *S. tritici*, *P. herpotrichoides* strains R and W, *M. fijiensis* and *M. musicola* were aligned (Fig. 1). The ITS sequences of *S. nodorum* and *S. avenae. triticea* were aligned (Fig. 2). An alignment was also made of the ITS sequences from *F. graminearum, F. culmorum, F. moniliforme* and *M. nivale* (Fig. 3). Sets of primers were synthesized according to EXAMPLE 5 based on analysis of the aligned sequences. Primers were designed to regions containing the greatest differences in sequence among the fungal species for Figs. 1-2. In Fig 3, primers were designed to regions of highest homology within the ITS for *Fusarium*. In addition, the published ribosomal gene-specific primers ITS1 (SEQ ID NO:38), ITS2 (SEQ ID NO:39), ITS3 (SEQ ID NO:40) and ITS4 (SEQ ID NO:41) (White *et al.*, 1990; In: PCR Protocols; Eds.: Innes *et al.* pages 315-322) were synthesized for testing in combination with the primers specific for the ITS region.

Table 2: Primer Design for Fungal Detection

Primer Template	Primer Name	Primer Sequence
S. nodorum	JB433	5' ACACTCAGTAGTTTACTACT 3' (SEQ ID NO:7)
S. nodorum	JB434	5' TGTGCTGCGCTTCAATA 3' (SEQ ID NO:8)
S. nodorum	JB525	5' GCGACTTGTGCTGCGCTTCAATA 3' (SEQ ID NO:9)

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S. nodorum	JB527	5' CATTACACTCAGTAGTTTACTACT 3' (SEQ ID NO:10)
S. tritici	JB445	5' CTGCGTCGGAGTTTACG 3' (SEQ ID NO:11)
S. tritici	JB446	5' CGAGGCTGGAGTGGTGT 3' (SEQ ID NO:12)
S. tritici	JB526	5' CCCAGCGAGGCTGGAGTGGTGT 3' (SEQ ID NO:13)
P. herp.	JB536	5' CTGGGGGCTACCCTACTTGGTAG 3' (SEQ ID NO:14)
P. herp.	JB537	5' GGGGGCTACCCTACTTGGTAG 3' (SEQ ID NO:15)
P. herp.	JB538	5' ACTTGGTAGGGTTTAGAGTCGTCA 3' (SEQ ID NO:16)
P. herp.	JB539	5' CTTCGGTAAGGTTTAGAGTCGTCG 3' (SEQ ID NO:17)
P. herp.	JB540	5' GGGGGCCACCCTACTTCGGTAA 3' (SEQ ID NO:18)
P. herp.	JB541	5' CCACTGATTTTAGAGGCCGCGAG 3' (SEQ ID NO:19)
P. herp.	JB542	5' CCACTGATTTTAGAGGCCGCGAA 3' (SEQ ID NO:20)
P. herp.	JB543	5' CCTGTAAAAAATTGGGGGTTA 3' (SEQ ID NO:21)
P. herp.	JB544	5' CCTGTAAAAAATTGGGGGTTG 3' (SEQ ID NO:22)
M. fijiensis	JB547	5' ATTACCGAGTGAGGGCTCACGC 3' (SEQ ID NO:23)
M. fijiensis	JB548	5' GTTGCTTCGGGGGCGACCTG 3' (SEQ ID NO:24)
M. fijiensis	JB442	5' TCGGGGGCGACCTGCCG 3' (SEQ ID NO:25)
M. fijiensis	JB443	5' CCGGAGGCCGTCTA 3' (SEQ ID NO:26)
M. fijiensis	JB545	5' CCACAACGCTTAGAGACGGACAG 3' (SEQ ID NO:27)
M. fijiensis	JB546	5' CACCCGCACTCCGAAGCGAATT 3' (SEQ ID NO:28)
M. fijiensis	JB549	5' GATCCGAGGTCAACCTTTGAATAA 3' (SEQ ID NO:29)
M. fijiensis	JB444	5' GGTCAACCTTTGAATAA 3' (SEQ ID NO:30)
M. musicola	JB451	5' CCTTTGTGAACCACACCT 3' (SEQ ID NO:31)
M. musicola	JB440	5' CTGCCGGCGAACTT 3' (SEQ ID NO:32)
M. musicola	JB449	5' ACCCTGCCGGCGAACTT 3' (SEQ ID NO:33)
M. musicola	JB448	5' GCGACCCTGCCGGCGAAC 3' (SEQ ID NO:34)
M. musicola	JB441	5' TAGCCGGGAGACTTTGG 3' (SEQ ID NO:35)
M. musicola	JB450	5' TCTGCGTCGGAGTTCC 3' (SEQ ID NO:36)
M. musicola	JB452	5' CCGCGCTCCGGAGCGAAC 3' (SEQ ID NO:37)
18S rDNA	ITS1	5' TCCGTAGGTGAACCTGCGG 3' (SEQ ID NO:38)
5.8S rDNA	ITS2	5' GCTGCGTTCTTCATCGATGC 3' (SEQ ID NO:39)
5.8S rDNA	ITS3	5' GCATCGATGAAGAACGCAGC 3' (SEQ ID NO:40)
25S rDNA	ITS4	5' TCCTCCGCTTATTGATATGC 3' (SEQ ID NO:41)
S. *nodorum*	JB563	5' CTTGCCTGCCGGTTGGACAAATT 3' (SEQ ID NO:50)
S. nodorum	JB564	5' CTCAGTAGTTTACTACTGTAAAAGG 3' (SEQ ID NO:51)
S. nodorum	JB565	5' CTTCTGGACGCAAGTGTTTGTTAC 3' (SEQ ID NO:52)
Fusarium spp.	JB566	5' GTTTTTAGTGGAACTTCTGAGT 3' (SEQ ID NO:53)
Fusarium spp.	JB567	5' CGCAGGAACCCTAAACTCT 3' (SEQ ID NO:54)
Fusarium spp.	JB568	5' GCCCGCCGCAGG 3' (SEQ ID NO:55)
Fusarium spp.	JB569	5' RTWWTTWRTGGAMYYTCTGAGT 3' (SEQ ID NO:56)
Fusarium spp.	JB570	5' TATGTTGCCTCGGCGG 3' (SEQ ID NO:57)
Fusarium spp.	JB571	5' TAACGATATGTAAATTACTACGCT 3' (SEQ ID NO:58)
Fusarium spp.	JB572	5' AAGTTGGGGTTTAACGGC 3' (SEQ ID NO:59)
Fusarium spp.	JB573	5' AGCGAGCCCGCCAC 3' (SEQ ID NO:60)
Fusarium spp.	JB574	5' CCATTGTGAACGTTACCTATAC 3' (SEQ ID NO:61)
Fusarium spp.	JB575	5' CGACCAGAGCGAGATGTA 3' (SEQ ID NO:62)
Fusarium spp.	JB576	5' GTGAACATACCTTATGTTGCC 3' (SEQ ID NO:63)
Fusarium spp.	JB577	5' GTTGCCTCGGCGGATC 3' (SEQ ID NO:64)
Fusarium spp.	JB578	5' CCGCGACGATTACCAG 3' (SEQ ID NO:65)

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NOTE: *Fusarium* spp. includes *F. graminearum, F. culmorum, F. moniliforme* and *Michrodochium nivale* (syn. *F. nivale*).

Example 7: Selection of Random Amplified Polymorphic DNA (RAPD) primers

Two RAPD primer libraries (kits B and E) of twenty oligonucleotides each were purchased from Operon Technologies Incorporated (Alameda, CA). The primers were tested for their ability to differentiate purified genomic DNA of *S. nodorum, S. tritici, M. fijiensis* and *M. musicola*. The PCR conditions were essentially the same as described in EXAMPLE 4 except the number of PCR cycles was increased to 35, the annealing temperature was 30°C and only 5 picamoles of each primer were used. Five RAPD primers were identified that differentiate purified genomic DNA of *S. nodorum, S. tritici, M. fijiensis* and *M. musicola*. Primers OPB-12 and OPE-6 produced a single fragment when amplified with *S. tritici* genomic DNA. Primers OPE-12, OPB-19 and OPE-15 produced single fragments from *S. nodorum* genomic DNA. Primers OPB-12 and OPE-6 did not produce any amplification products from *S. nodorum M. fijiensis* and *M. musicola* genomic DNA. Primers OPE-12, OPB-19 and OPE-15 did not amplify any fragments from genomic *S. tritici, M. fijiensis* or *M. musicola* DNA.

Table 3: RAPD Primers for *Septoria* Diagnosis

Source of template DNA	Primer	Sequence of primer	Approximate size of amplified fragment
S. tritici	OPB-12	5'-CCTTGACGCA-3' (SEQ ID NO: 42)	1.3 kb
S. tritici	OPE-6	5'-AAGACCCCTC-3' (SEQ ID NO: 43)	1.0 kb
S. nodorum	OPE-12	5'-TTATCGCCCC-3' (SEQ ID NO: 44)	2.2 kb
S. nodorum	OPB-19	5'-ACCCCCGAAG-3' (SEQ ID NO: 45)	1.1 kb
S. nodorum	OPE-15	5'-ACGCACAACC-3' (SEQ ID NO: 46)	1.3 kb

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Example 8: Determination of primer specificity to purified fungal genomic DNA

PCRs were performed according to EXAMPLE 4 using different primer combinations in an attempt to amplify a single species-specific fragment. Species-specific PCR amplification products were produced from primers designed from the ITS region between the 18S and 25S ribosomal DNA subunits of each fungal strain of interest.

Table 4: ITS-derived diagnostic PCR primers

Source of template DNA	5'Primer	3'Primer	Approximate size of amplified fragment
Septoria nodorum	JB433 (SEQ ID NO:7)	JB434 (SEQ ID NO:8)	448bp
	JB433 (SEQ ID NO:7)	ITS4 (SEQ ID NO:41)(JB415)	553bp
	ITS1 (SEQ ID NO:38)(JB410)	JB434 (SEQ ID NO:8)	478bp
	ITS3 (SEQ ID NO:40)(JB414)	JB434 (SEQ ID NO:8)	232bp*
	JB527 (SEQ ID NO:10)	JB525 (SEQ ID NO:9)	458bp
	JB564 (SEQ ID NO:51)	JB565 (SEQ ID NO:52)	480bp
	JB563 (SEQ ID NO:50)	JB565 (SEQ ID NO:52)	368bp
Septoria tritici	JB445 (SEQ ID NO:11)	ITS4 (SEQ ID NO:41)(JB415)	407bp
	ITS1 (SEQ ID NO:38)(JB410)	JB446 (SEQ ID NO:12)	345bp
	ITS3 (SEQ ID NO:40)(JB414)	JB446 (SEQ ID NO:12)	143bp*
	JB445 (SEQ ID NO:11)	JB446 (SEQ ID NO:12)	204bp
*			
M. fijiensis	JB443 (SEQ ID NO:26)	ITS4 (SEQ ID NO:41)(JB415)	418bp
	ITS1 (SEQ ID NO:38)(JB410)	JB444 (SEQ ID NO:30)	482bp
	JB443 (SEQ ID NO:26)	JB444 (SEQ ID NO:30)	366bp*
	ITS3 (SEQ ID NO:40)(JB414)	JB444 (SEQ ID NO:30)	281bp*
	ITS1 (SEQ ID NO:38)(JB410)	JB549 (SEQ ID NO:29)	489bp
M. musicola	JB449 (SEQ ID NO:33)	ITS4 (SEQ ID NO:41)(JB415)	430bp
	JB448 (SEQ ID NO:34)	ITS4 (SEQ ID NO:41)(JB415)	449bp*
	JB448 (SEQ ID NO:34)	ITS2 (SEQ ID NO:39)(JB411)	138bp*
	JB450 (SEQ ID NO:36)	ITS4 (SEQ ID NO:41)(JB415)	390bp*

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P. herpotrichoides

JB536 (SEQ ID NO:14)	JB541 (SEQ ID NO:19)	415bp[+]
JB536 (SEQ ID NO:14)	JB543 (SEQ ID NO:21)	502bp[+]
JB537 (SEQ ID NO:15)	JB541 (SEQ ID NO:19)	413bp[+]
JB537 (SEQ ID NO:15)	JB543 (SEQ ID NO:21)	500bp[+]
JB538 (SEQ ID NO:16)	JB541 (SEQ ID NO:19)	401bp[+]
JB538 (SEQ ID NO:16)	JB543 (SEQ ID NO:21)	488bp[+]
JB536 (SEQ ID NO:14)	ITS4 (SEQ ID NO:41)(JB415)	560bp[+]
JB537 (SEQ ID NO:15)	ITS4 (SEQ ID NO:41)(JB415)	558bp[+]
JB538 (SEQ ID NO:16)	ITS4 (SEQ ID NO:41)(JB415)	546bp[+]
ITS1 (SEQ ID NO:38)(JB410)	JB541 (SEQ ID NO:19)	482bp[+]
ITS1 (SEQ ID NO:38)(JB410)	JB543 (SEQ ID NO:21)	569bp[+]
ITS1 (SEQ ID NO:38)(JB410)	JB542 (SEQ ID NO:20)	482bp[+]
ITS1 (SEQ ID NO:38)(JB410)	JB544 (SEQ ID NO:22)	569bp[++]
JB540 (SEQ ID NO:18)	ITS4 (SEQ ID NO:41)(JB415)	558bp[++]
JB539 (SEQ ID NO:17)	ITS4 (SEQ ID NO:41)(JB415)	545bp[++]
JB540 (SEQ ID NO:18)	JB542 (SEQ ID NO:20)	413bp[++]
JB540 (SEQ ID NO:18)	JB544 (SEQ ID NO:22)	500bp[++]
JB539 (SEQ ID NO:17)	JB542 (SEQ ID NO:20)	400bp[++]
JB539 (SEQ ID NO:17)	JB544 (SEQ ID NO:22)	487bp[++]

Fusarium spp.

JB566 (SEQ ID NO:53)	ITS4 (SEQ ID NO:41)(JB415)	430bp[1]
JB566 (SEQ ID NO:53)	JB572 (SEQ ID NO:59)	346bp[1]
JB569 (SEQ ID NO:56)	ITS4 (SEQ ID NO:41)(JB415)	430bp[1]
JB569 (SEQ ID NO:56)	JB572 (SEQ ID NO:59)	346bp[1]
ITS1 (SEQ ID NO:38)(JB410)	JB572 (SEQ ID NO:59)	485bp[1]
JB566 (SEQ ID NO:53)	JB571 (SEQ ID NO:58)	308bp[2]
JB569 (SEQ ID NO:56)	JB571 (SEQ ID NO:58)	308bp[2]
JB570 (SEQ ID NO:57)	ITS4 (SEQ ID NO:41)(JB415)	501bp[2]
JB570 (SEQ ID NO:57)	JB571 (SEQ ID NO:58)	379bp[2]
JB570 (SEQ ID NO:57)	JB578 (SEQ ID NO:65)	395bp[2]
JB567 (SEQ ID NO:54)	ITS4 (SEQ ID NO:41)(JB415)	450bp[2]
JB567 (SEQ ID NO:54)	JB571 (SEQ ID NO:58)	328bp[2]
JB567 (SEQ ID NO:54)	JB572 (SEQ ID NO:59)	366bp[2]
JB567 (SEQ ID NO:54)	JB578 (SEQ ID NO:65)	344bp[2]
JB568 (SEQ ID NO:55)	ITS4 (SEQ ID NO:41)(JB415)	459bp[2]
JB568 (SEQ ID NO:55)	JB571 (SEQ ID NO:58)	337bp[2]
JB568 (SEQ ID NO:55)	JB572 (SEQ ID NO:59)	375bp[2]
JB576 (SEQ ID NO:63)	ITS4 (SEQ ID NO:41)(JB415)	510bp[2]
JB576 (SEQ ID NO:63)	JB578 (SEQ ID NO:65)	404bp[2]
JB577 (SEQ ID NO:64)	ITS4 (SEQ ID NO:41)(JB415)	495bp[2]
JB577 (SEQ ID NO:64)	JB571 (SEQ ID NO:58)	373bp[2]
JB577 (SEQ ID NO:64)	JB578 (SEQ ID NO:65)	389bp[2]
ITS1 (SEQ ID NO:38)(JB410)	JB571 (SEQ ID NO:58)	447bp[2]
ITS1 (SEQ ID NO:38)(JB410)	JB578 (SEQ ID NO:65)	463bp[2]
ITS1 (SEQ ID NO:38)(JB410)	JB575 (SEQ ID NO:62)	479bp[2]

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M. nivale			
	JB569 (SEQ ID NO:56)	JB575 (SEQ ID NO:62)	340bp
	JB567 (SEQ ID NO:54)	JB575 (SEQ ID NO:62)	360bp
	JB574 (SEQ ID NO:61)	ITS4 (SEQ ID NO:41)(JB415)	520bp
	JB574 (SEQ ID NO:61)	JB572 (SEQ ID NO:59)	436bp

*...Primer combination amplified some fragments by false priming but none were the size of the desired fragment.

+....Primers amplified the correct size fragment from both R-type and W-type of *Pseudocercosporella herpotrichoides*.

++...Primer combination amplified the correct size fragment from the R-type of *P. herpotrichoides* only.

[1]....Primer combination amplified the correct size fragment from *F. graminearum, F. culmorum, F. moniliforme* and *M. nivale*.

[2]....Primer combination amplified the correct size fragment from *F. graminearum, F. culmorum* and *F. moniliforme*.

Example 9: Determination of primer specificity to plant tissue infected with fungi

Total genomic DNA was isolated from healthy wheat leaves, wheat leaves infected with *S. nodorum*, wheat leaves infected with *S. tritici* and wheat leaves infected with both *S. nodorum* and *S. tritici* using the protocol described in EXAMPLE 3. PCRs were performed as described in EXAMPLE 4 testing the primer combinations listed in EXAMPLE 8 against DNA from the wheat leaves.

The *S. tritici*-specific primer JB446 (SEQ ID NO:12) and ITS1 (SEQ ID NO:38)(JB410) amplified a 345 bp fragment from purified *S. tritici* DNA, from *S. tritici*-infected wheat leaf tissue and from a wheat leaf sample infected with both *S. tritici* and *S. nodorum*. The primer set did not amplify a diagnostic fragment from healthy wheat leaf tissue nor from *S. nodorum*-infected wheat tissue. Similarly, the *S. tritici*-specific primers JB445 (SEQ ID NO:11) and ITS4 (SEQ ID NO:41)(JB415) amplified a 407 bp fragment from the same tissues as the primer combination JB446 (SEQ ID NO:12) and ITS1 (SEQ ID NO:38)(JB410) and was also diagnostic.

Similarly diagnostic results were obtained with the *S. nodorum*-specific primers JB433 (SEQ ID NO:7) and JB434 (SEQ ID NO:8). The primers amplified a 448 bp fragment from *S. nodorum*-infected wheat tissue, from a wheat leaf sample infested with both *S. nodorum* and *S. tritici*, as well as from purified genomic DNA of *S. nodorum*. The primer combination

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JB433 (SEQ ID NO:7) and JB434 (SEQ ID NO:8) did not amplify any fragments from healthy wheat tissue, from *S.tritici*-infected wheat tissue or from purified genomic DNA of *S. tritici*. The *S. nodorum*-specific primers JB527 (SEQ ID NO:10) and JB525 (SEQ ID NO:9) amplified a 458 bp fragment from the same genomic DNAs and wheat tissues as the JB433 (SEQ ID NO:7) and JB434 (SEQ ID NO:8) combination.

The P. herpotrichoides primer combinations listed in EXAMPLE 8 were PCR tested against the extracts from wheat stems as pbtained in Example 12. PCRs were performed as described in EXAMPLE 4 with the following changes: 35 cycles were run of 94°C for 15 sec and 70°C for 45 sec, 1.5 - 2.5 mM MgCl2 and 200 µM of each dNTP was used. 1 µl of wheat extract was used in each PCR.

Primer combination JB537 (SEQ ID NO:15) and JB541 (SEQ ID NO:19) amplified a 413 bp fragment from wheat extract infected with the W-type pathotype of *P. herpotrichoides*. No amplification products were produced from amplification with healthy wheat extract nor from wheat extract infected with the R-type pathotype of *P. herpotrichoides*.

The primer combination JB539 (SEQ ID NO:17) and JB544 (SEQ ID NO:22) amplified a 487 bp fragment and primer combination JB540 (SEQ ID NO:18) and JB542 (SEQ ID NO:20) amplified a 413 bp fragment from R-type infected wheat but not from healthy wheat nor from W-type infected wheat.

Total genomic DNA was also isolated from healthy banana leaves and from banana leaves infected with *M. fijiensis* using the protocol described in EXAMPLE 3. PCRs were performed as described in EXAMPLE 4 testing the *M. fijiensis* primer combinations listed in EXAMPLE 8 against DNA from the banana leaves.

The *M. fijiensis*-specific primer JB549 (SEQ ID NO:29) and ITS1 (SEQ ID NO:38)(JB410) amplified a 489 bp fragment from purified *M. fijiensis* DNA and from *M. fijiensis*-infected banana leaf tissue. The primer set did not amplify a diagnostic fragment from healthy banana leaf tissue. The *M. fijiensis*-specific primer combinations JB443 (SEQ ID NO:26)/ITS4 (SEQ ID NO:41)(JB415) and ITS1 (SEQ ID NO:38)(JB410)/JB444 (SEQ ID NO:30) amplified a 418 bp fragment and a 482 bp fragment, respectively, from the same genomic

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DNA and banana leaf tissue as the JB549 (SEQ ID NO:29) and ITS1 (SEQ ID NO:38)(JB410) primer combination.

Example 10: Determination of cross-reactivity of species-specific primers with other species and isolates

Purified fungal genomic DNAs were obtained as described in EXAMPLE 1 and PCR assayed as described in EXAMPLE 4 using the species-specific primers. Other fungal DNA species and isolates were tested for the species-specific primers ability to cross-react with them.

The *S. tritici*-specific primer JB446 (SEQ ID NO:12) and ITS1 (SEQ ID NO:38)(JB410) amplified a 345 bp fragment from all of the *S. tritici* isolates listed in EXAMPLE 1. There was no cross-reactivity with purified genomic DNA of *S. nodorum, S. glycines* or *S. passerini*. None of these other fungal species produced an amplification product with the *S. tritici*-specific primers.

A 448 bp fragment was amplified from all of the *S. nodorum* isolates listed in EXAMPLE 1 using the *S. nodorum*-specific primers JB433 (SEQ ID NO:7) and JB434 (SEQ ID NO:8). Similarly the *S. nodorum*-specific primers JB527 (SEQ ID NO:10) and JB525 (SEQ ID NO:9) amplified a 458 bp fragment from all the *S. nosorum* isolates listed in EXAMPLE 1. S. *tritici, S. glycines* and *S. passerini* did not produce any amplification products when assayed with the either of the *S. nodorum*-specific primer sets JB433 (SEQ ID NO:7) and JB434 (SEQ ID NO:8) or JB527 (SEQ ID NO:10) and JB525 (SEQ ID NO:9).

PCRs were run using the conditions described in EXAMPLE 9, the *P. herpotrichoides*-specific primer combinations listed in EXAMPLE 8 against the other fungal DNA species and isolates listed in EXAMPLE 1.

The primer combination JB537 (SEQ ID NO:15) and JB541 (SEQ ID NO:19) produced a 413 bp fragment from the W-type *P. herpotrichoides* isolates only when tested against the *P. herpotrichoides* isolates and the following cereal pathogens: *P. aestiva, C. cereale, P. sorokiniana, S. tritici* and *S. nodorum*. The primer combiantion JB539 (SEQ ID NO:17) and

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JB544 (SEQ ID NO:22) amplified a 487 bp fragment from the R-type *P. herpotrichoides* isolate only when tested against the same DNAs. The primer combination JB540 (SEQ ID NO:18) and JB542 (SEQ ID NO:20) produced a 413 bp fragment from the R-type *P. herpotrichoides* isolate only when tested against the same DNAs.

Example 11: Sources of Pseudocercosporella herpotrichoides-infected wheat

Eyespot-infected wheat stems were received from the stage 1c fungicide screening program of Ciba Basle. Eight day old wheat plants were infected with *P. herpotrichoides* by spraying a conidial suspension ($5x10^5$ conidia/ml) in 0.2% Tween 20 on the base of the wheat stems. After inoculation, the plants were covered with plastic and incubated for one day at 20°C and 95-100% relative humidity. The plants were transferred to a growth chamber where they were incubated for four weeks at 12°C and 60% relative humidity. After this incubation, the plants were moved to a greenhouse and incubated at 18°C and 60% relative humidity. Wheat plants infected with W-type *P. herpotrichoides* strain 311 were sampled at 8-9 weeks post-infection, while those infected with the R-type strain 308 pathogen were harvested at 9-10 weeks post-infection

Example 12: DNA extraction from wheat stems for *P. herpotrichoides* assay

DNA was extracted from wheat stems using the protocol described by Klimyuk *et al.* (The Plant Journal 3(3):493-494) with some modifications. A 2 cm wheat stem cut 0.5 cm above the basal culm was placed in 160 µl of 0.25 M NaOH and ground with a Kontes pestle until completely macerated. The sample was boiled for 30 s. 160 µl of 0.25 M HCl and 80 µl of 0.5 M Tris-Cl,pH8.0/ 0.25% v/v Nonidet P-40 were added to the sample. The sample was boiled for an additional 2 mins., then placed in an ice water bath. 1 µl of extract was used in the PCR assay.

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Example 13: Incorporation of diagnostic assays into a quantitative colormetric assay format

The colormetric assay was performed according to Nikiforov *et al.* (PCR Methods and Applications 3:285-291) with the following changes:

1) 30 µl of the R-type PCR product and 3 M NaCl/20 mM EDTA mixture were added to the capture primer well. 50 µl of the W-type PCR product and 3 M NaCl/20 mM EDTA mixture were used in the hybridization reaction.

2) The exonuclease treatment and hybridization reaction were incubated at 37°C.

3) A 1:1000 dilution of anti-biotin horseradish peroxidase (HRP) monoclonal antibody was used.

4) After a 2 min. incubation with the O-phenylenediamine dihydrochloride (OPD) substrate, 50 µl of 3 N HCl were added to each assay well. 96-well plates were read at 492 nm and referenced at 570 nm using a conventional ELISA plate reader.

The primers listed in Table 5 were synthesized as described in EXAMPLE 5 for testing as capture primers for the colormetric assay.

Table 5: Capture Primer Design for Colormetric Assay

Primer Name	Primer Template	Primer Sequence
ITS2	5.8S rDNA	5'GCTGCGTTCTTCATCGATGC3' (SEQ ID NO:39)
JB541	W-type *P. herp.*	5'CCACTGATTTTAGAGGCCGCGAG3'(SEQ ID NO:19)
JB542	R-type *P. herp.*	5'CCACTGATTTTAGAGGCCGCGAA3'(SEQ ID NO:20)
JB538'	W-type *P. herp.*	5'TGACGACTCTAAACCCTACCA3' (SEQ ID NO:66)
JB539'	R-type *P. herp.*	5'CGACGACTCTAAACCTTACCG3' (SEQ ID NO:67)
W130	W-type *P. herp.*	5'ATTCAAGGGTGGAGGTCTGA3' (SEQ ID NO:68)
R130	R-type *P. herp.*	5'ATTCAAGGGTGGAGGTCTGG3' (SEQ ID NO:69)
JB538'15	W-type *P. herp.*	5'CTCTAAACCCTACCA3' (SEQ ID NO:70)
JB539'15	R-type *P. herp.*	5'CTCTAAACCTTACCG3' (SEQ ID NO:71)
JB553	R & W types	5'GTGGTCCTCTGGCAG3' (SEQ ID NO:72)

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JB554 R & W types	5'CTCAACAGCCGAAGC3' (SEQ ID NO:73)
JB555 W-type *P. herp.*	5'GGGTGGAGGTCTGA3' (SEQ ID NO:74)
JB556 R-type *P. herp.*	5'GGTGGAGGTCTGG3' (SEQ ID NO:75)
JB561 R-type *P. herp.*	5'TGGAGGTCTGGACCA3' (SEQ ID NO:76)
JB562 W-type *P. herp.*	5'TGGAGGTCTGAACCA3' (SEQ ID NO:77)
JB559 W-type *P. herp.*	5'AGGGTGGAGGTCTGA3' (SEQ ID NO:78)
JB560 R-type *P. herp.*	5'AGGGTGGAGGTCTGG3' (SEQ ID NO:79)
JB557 W-type *P. herp.*	5'TTCTCCGAGAGGCCT3' (SEQ ID NO:80)
JB558 R-type *P. herp.*	5'TTCTCCGAGAGGCCC3' (SEQ ID NO:81)

The *S. nodorum* diagnostic primers JB527 (SEQ ID NO:10) and JB525 (SEQ ID NO:9) were integrated into the quantitative colormetric assay format. The primer JB527 (SEQ ID NO:10) was synthesized by Midland Certified Reagent Complany (Midland, Texas) to contain a biotin label and the 5' end to contain four internucleotidic phosphorothioate bonds. PCR amplification as described in EXAMPLE 4 using the modified JB527 (SEQ ID NO:10) and JB525 (SEQ ID NO:9) primers from healthy, low, medium, and highly *S. nodorum*-infected wheat produced no, low, medium and high A492 values, respectively, when assayed colormetrically using the ITS2 (SEQ ID NO:39) primer as the PCR product capture primer.

The *P. herpotrichoides* R-type specific 5' primers, JB539 (SEQ ID NO:17) and JB540 (SEQ ID NO:18), and the *P. herpotrichoides* W-type specific 5' primer, JB537 (SEQ ID NO:15), were also modified to contain a biotin label and four internucleotidic phosphorothioate bonds. A colormetric version of the *P. herpotrichoides* R-type PCR assay was developed using the modified JB540 (SEQ ID NO:18) primer, JB542 (SEQ ID NO:20) primer and the capture primer JB539'15. The products produced from amplification from R-type infected wheat and from R-type genomic DNA using the modified JB540 (SEQ ID NO:18) primer and JB542 (SEQ ID NO:20) primer produced positive colormetric values when assayed colormetrically. Positive colormetric values were also obtained by colormetric analysis of the PCR products from amplification using the modified JB537 (SEQ ID NO:15) primer and W-type specific primer JB541 (SEQ ID NO:19)with W-type infected wheat and W-type genomic DNA when JB538'15 was used as the capture primer. Furthermore, the intensity of the colormetric signal corresponded to the fragment intensity of the PCR product as visualized on an agarose gel.

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Previously, the different *Septoria* species were identifiable by examination under the microscope, and the identification of the different *Pseudocercosporella* strains has been possible only by pathological tests. Similarly, the unambiguous identification of *Mycosphaerella musicola* and *Mycosphaerella fijiensis* has been difficult, and even the isolation of mature perithecia does not always allow accurate identification (Pons, 1990; In: Sigatoka Leaf Spot Diseases of Banana, Eds. RA Fullerton and RH Stover, International Network for the Improvement of Banana and Plantain, France). Currently immunodiagnostic kits utilizing ELISA technology are routinely used to identify *Septoria tritici, Septoria nodorum, Pseudocercosporella herpotrichoides* and other pathogen, but this technology lacks the accuracy, detection limit and ability to distinguish different isolates of the instant invention. In consequence, the development of a DNA test for the rapid identification of different strains of these fungi offers real advantages not only to fungal taxonomists, but also for disease management and selective fungicide use in the field.

While the present invention has been described with reference to specific embodiments thereof, it will be appreciated that numerous variations, modifications, and further embodiments are possible, and accordingly, all such variations, modifications and embodiments are to be regarded as being within the scope of the present invention.

Deposits

The following deposits were made on March 28, 1994, at Agricultural Research Service, Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 North University Street, Peoria, Illinois 61604, U.S.A.:

1.	HB101 DH5d (pCRW2-1; SEQ ID NO: 3)	Accession No. NRRL B-21231
2.	HB101 DH5d (pCRW5-1; SEQ ID. NO: 47)	Accession No. NRRL B-21232
3.	E. coli DH5d (pCRSTRIT1; SEQ ID NO: 1)	Accession No. NRRL B-21233
4.	E. coli DH5d (pCRR1-21; SEQ ID NO: 4)	Accession No. NRRL B-21234
5.	E. coli DH5d (pCRSNOD31; SEQ ID NO: 2)	Accession No. NRRL B-21235

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Ligon, James M
Beck, James J

(ii) TITLE OF INVENTION: Detection of Fungal Pathogens Using the Polymerase Chain Reaction

(iii) NUMBER OF SEQUENCES: 86

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(E) COUNTRY: USA
(F) ZIP: 10532

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US TBA
(B) FILING DATE:
(C) CLASSIFICATION:

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(B) FILING DATE: 04-APR-1994

(viii) ATTORNEY/AGENT INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 548 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..548
(D) OTHER INFORMATION: /note= "DNA sequence for the Internal Transcribed Spacer of Septoria tritici"

3NSDOCID: <WO___9529260A2_I_>

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TCCGTAGGTG AACCTGCGGA GGGATCATTA CCGAGCGAGG GCCTCCGGGT CCGACCTCCA 60

ACCCTTTGTG AACACATCCC GTTGCTTCGG GGGCGACCCT GCCGGGCGCC CCCGGAGGAC 120

CACCAAAAAA CACTGCATCT CTGCGTCGGA GTTTACGAGT AAATCGAAAC AAAACTTTCA 180

ACAACGGATC TCTTGGTTCT GGCATCGATG AAGAACGCAG CGAAATGCGA TAAGTAATGT 240

GAATTGCAGA ATTCAGTGAA TCATCGAATC TTTGAACGCA CATTGCGCCC CCTGGTATTC 300

CGGGGGGCAT GCCCGTTCGA GCGTCATTAC ACCACTCCAG CCTCGCTGGG TATTGGGCGT 360

CTTTTCGCGG GGGATCACTC CCCCGCGCGC CTCAAAGTCT CCGGCTGAGC GGTCTCGTCT 420

CCCAGCGTTG TGGCATCACG TCTCGCCGCG GAGTTCACGA GCCCTCACGG CCGTTAAATC 480

ACACCTCAGG TTGACCTCGG ATCGGGTAGG GATACCCGCT GAACTTAAGC ATATCAATAA 540

GCGGAGGA 548

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 583 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Septoria nodorum

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..583
(D) OTHER INFORMATION: /note= "DNA sequence for the Internal Transcribed Spacer of Septoria nodorum"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TCCGTAGGTG AACCTGCGGA AGGATCATTA CACTCAGTAG TTTACTACTG TAAAAGGGGC 60

TGTTAGTCTG TATAGCGCAA GCTGATGAGC AGCTGGCCTC TTTTATCCAC CCTTGTCTTT 120

TGCGTACCCA CGTTTCCTCG GCAGGCTTGC CTGCCGGTTG GACAAATTTA TAACCTTTTT 180

AATTTTCAAT CAGCGTCTGA AAAACTTAAT AATTACAACT TTCAACAACG GATCTCTTGG 240

TTCTGGCATC GATGAAGAAC GCAGCGAAAT GCGATAAGTA GTGTGAATTG CAGAATTCAG 300

TGAATCATCG AATCTTTGAA CGCACATTGC GCCCCTTGGT ATTCCATGGG GCATGCCTGT 360

TCGAGCGTCA TTTGTACCCT CAAGCTCTGC TTGGTGTTGG GTGTTTGTCC TCTCCCTAGT 420

GTTTGGACTC GCCTTAAAAT AATTGGCAGC CAGTGTTTTG GTATTGAAGC GCAGCACAAG 480

TCGCGATTCG TAACAAACAC TTGCGTCCAC AAGCCTTTTT AACTTTTGAC CTCGGATCAG 540

GTAGGGATAC CCGCTGAACT TAAGCATATC AATAAGCGGA GGA 583

BNSDOCID: <WO___9529260A2_I_>

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 626 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Pseudocercosporella herpotrichoides
(B) STRAIN: Strain R
(C) INDIVIDUAL ISOLATE: Variant W2-1

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..626
(D) OTHER INFORMATION: /note= "DNA sequence for the Internal Transcribed Spacer of Pseudocercosporella herpotrichoides strain W (variant W2-1)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TCCGTAGGTG AACCTGCGGA AGGATCATTA ATAGAGCAAT GAACAGACAG CGCCCCGGGA 60

GAAATCCTGG GGGCTACCCT ACTTGGTAGG GTTTAGAGTC GTCAGGCCGC TCGGAGAAGC 120

CTGGTTCAGA CCTCCACCCT TGAATAAATT ACCTTTGTTG CTTTGGCAGG GCGCCTCGCG 180

CCAGCGGCTT CGGCTGTTGA GTACCTGCCA GAGGACCACA ACTCTTGTTT TTAGTGATGT 240

CTGAGTACTA TATAATAGTT AAAACTTTCA ACAACGGATC TCTTGGTTCT GGCATCGATG 300

AAGAACGCAG CGAAATGCGA TAAGTAATGT GAATTGCAGA ATTCAGTGAA TCATCGAATC 360

TTTGAACGCA CATTGCGCCC TCTGGTATTC CGGGGGGCAT GCCTGTTCGA GCGTCATTAT 420

AACCACTCAA GCTCTCGCTT GGTATTGGGG TTCGCGTCCT CGCGGCCTCT AAAATCAGTG 480

GCGGTGCCTG TCGGCTCTAC GCGTAGTAAT ACTCCTCGCG ATTGAGTCCG GTAGGTTTAC 540

TTGCCAGTAA CCCCCAATTT TTTACAGGTT GACCTCGGAT CAGGTAGGGA TACCCGCTGA 600

ACTTAAGCAT ATCAATAAGC GGAGGA 626

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 627 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Pseudocercosporella herpotrichoides
(B) STRAIN: Strain R

BNSDOCID: <WO__9529260A2_I_>

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..627
(D) OTHER INFORMATION: /note= "DNA sequence for the Internal Transcribed Spacer of Pseudocercosporella herpotrichoides Strain R"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TCCGTAGGTG AACCTGCGGA AGGATCATTA ATAGAGCAAT GGATAGACAG CGCCCCGGGA 60

GAAATCCTGG GGGCCACCCT ACTTCGGTAA GGTTTAGAGT CGTCGGGCCT CTCGGAGAAG 120

CCTGGTCCAG ACCTCCACCC TTGAATAAAT TACCTTTGTT GCTTTGGCAG GGCGCCTCGC 180

GCCAGCGGCT TCGGCTGTTG AGTACCTGCC AGAGGACCAC AACTCTTGTT TTTAGTGATG 240

TCTGAGTACT ATATAATAGT TAAAACTTTC AACAACGGAT CTCTTGGTTC TGGCATCGAT 300

GAAGAACGCA GCGAAATGCG ATAAGTAATG TGAATTGCAG AATTCAGTGA ATCATCGAAT 360

CTTTGAACGC ACATTGCGCC CTCTGGTATT CCGGGGGGCA TGCCTGTTCG AGCGTCATTA 420

TAACCACTCA AGCTCTCGCT TGGTATTGGG GTTCGCGTCT TCGCGGCCTC TAAAATCAGT 480

GGCGGTGCCT GTCGGCTCTA CGCGTAGTAA TACTCCTCGC GATTGAGTCC GGTAGGTTTA 540

CTTGCCAGCA ACCCCCAATT TTTTACAGGT TGACCTCGGA TCAGGTAGGG ATACCCGCTG 600

AACTTAAGCA TATCAATAAG CGGAGGA 627

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 534 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Mycosphaerella fijiensis

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..534
(D) OTHER INFORMATION: /note= "DNA sequence for the Internal Transcribed Spacer of Mycosphaerella fijiensis"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TCCGTAGGTG AACCTGCGGA GGGATCATTA CCGAGTGAGG GCTCACGCCC GACCTCCAAC 60

CCTTTGTGAA CCACAACTTG TTGCTTCGGG GGCGACCTGC CGTCGGCGGG CGCCCCCGGA 120

GGCCGTCTAA ACACTGCATC TTTGCGTCGG AGTTTAAAAC AAATCGAACA AAACTTTCAA 180

CAACGGATCT CTTGGTTCTG GCATCGATGA AGAACGCAGC GAAATGCGAT AAGTAATGTG 240

AATTGCAGAA TTCAGTGAAT CATCGAATCT TTGAACGCAC ATTGCGCCCT TTGGTATTCC 300

BNSDOCID: <WO___9529260A2_I_>

GAAGGGCATG CCTGTTCGAG CGTCATTTCA CCACTCAAGC CTGGCTTGGT ATTGGGCGTC 360

GCGGTTCTTC GCGCGCCTTA AAGTCTCCGG CTGAGCTGTC CGTCTCTAAG CGTTGTGGAT 420

CTTTCAATTC GCTTCGGAGT GCGGGTGGCC GCGGCCGTTA AATCTTTATT CAAAGGTTGA 480

CCTCGGATCA GGTAGGGATA CCCGCTGAAC TTAAGCATAT CAATAAGCGG AGGA 534

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 540 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Mycosphaerella musicola

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..540
(D) OTHER INFORMATION: /note= "DNA sequence for the Internal Transcribed Spacer of Mycosphaerella musicola"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TCCGTAGGTG AACCTGCGGG GGGATCATTA CCGAGTGAGG GCTCACCCCC GACCTCCAAC 60

CCTTTGTGAA CCACACCTGT TGCTTCGGGG GCGACCCTGC CGGCGAACTT GTCGCCGGGC 120

GCCCCCGGAG GTCTCCTTAA CACTGCATCT CTGCGTCGGA GTTCCAAACA AATCGGACAA 180

AACTTTCAAC AACGGATCTC TTGGTTCTGG CATCGATGAA GAACGCAGCG AAATGCGATA 240

AGTAATGTGA ATTGCAGAAT TCAGTGAATC ATCGAATCTT TGAACGCACA TTGCGCCCTT 300

TGGCATTCCG AAGGGCATGC CTGTTCGAGC GTCATTTCAC CACTCAAGCC TAGCTTGGTA 360

TTGGGCGCCG CGGTGCTCCG CGCGCCCCAA AGTCTCCCGG CTAAGCCGTC CGTCTCTAAG 420

CGTTGTGGAT TTTTCAGTTC GCTCCGGAGC GCGGGTGGCC GCGGCCGTTA AATCTTCAAA 480

GGTTGACCTC GGATCAGGTA GGGATACCCG CTGAACTTAA GCATATCAAT AAGCGGAGGA 540

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer JB433

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

INSDOCID: <WO___9529260A2_I_>

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ACACTCAGTA GTTTACTACT 20

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer JB434

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TGTGCTGCGC TTCAATA 17

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer JB525

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GCGACTTGTG CTGCGCTTCA ATA 23

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer JB527

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

BNSDOCID: <WO__9529260A2_I_>

CATTACACTC AGTAGTTTAC TACT 24

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer JB445

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CTGCGTCGGA GTTTACG 17

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer JB446

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CGAGGCTGGA GTGGTGT 17

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer JB526

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CCCAGCGAGG CTGGAGTGGT GT 22

(2) INFORMATION FOR SEQ ID NO:14:

BNSDOCID: <WO___9529260A2_I_>

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer JB536

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CTGGGGGCTA CCCTACTTGG TAG 23

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer JB537

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GGGGGCTACC CTACTTGGTA G 21

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer JB538

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

ACTTGGTAGG GTTTAGAGTC GTCA 24

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

BNSDOCID: <WO__9529260A2_I_>

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer JB539

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CTTCGGTAAG GTTTAGAGTC GTCG 24

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer JB540

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GGGGGCCACC CTACTTCGGT AA 22

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer JB541

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CCACTGATTT TAGAGGCCGC GAG 23

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer JB542

(iii) HYPOTHETICAL: NO

INSDOCID: <WO___9529260A2_I_>

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CCACTGATTT TAGAGGCCGC GAA 23

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer JB543

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CCTGTAAAAA ATTGGGGGTT A 21

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer JB544

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CCTGTAAAAA ATTGGGGGTT G 21

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer JB547

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

BNSDOCID: <WO___9529260A2_I_>

ATTACCGAGT GAGGGCTCAC GC 22

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer JB548

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GTTGCTTCGG GGGCGACCTG 20

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer JB442

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

TCGGGGGCGA CCTGCCG 17

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer JB443

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CCGGAGGCCG TCTA
14

(2) INFORMATION FOR SEQ ID NO:27:

BNSDOCID: <WO___9529260A2_I_>

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer JB545

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CCACAACGCT TAGAGACGGA CAG 23

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer JB546

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CACCCGCACT CCGAAGCGAA TT 22

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer JB549

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

GATCCGAGGT CAACCTTTGA ATAA 24

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs

BNSDOCID: <WO__9529260A2_I_>

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer JB444

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GGTCAACCTT TGAATAA 17

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer JB451

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

CCTTTGTGAA CCACACCT 18

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer JB440

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CTGCCGGCGA ACTT 14

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

3NSDOCID: <WO___9529260A2_I_>

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer JB449

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

ACCCTGCCGG CGAACTT 17

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer JB448

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

GCGACCCTGC CGGCGAAC 18

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer JB441

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

TAGCCGGGAG ACTTTGG 17

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer JB450

BNSDOCID: <WO___9529260A2_I_>

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

TCTGCGTCGG AGTTCC 16

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer JB452

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

CCGCGCTCCG GAGCGAAC 18

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer ITS1

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

TCCGTAGGTG AACCTGCGG 19

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer ITS2

(iii) HYPOTHETICAL: NO

BNSDOCID: <WO___9529260A2_I_>

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

GCTGCGTTCT TCATCGATGC 20

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer ITS3

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

GCATCGATGA AGAACGCAGC 20

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer ITS4

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

TCCTCCGCTT ATTGATATGC 20

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer OPB-12

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

BNSDOCID: <WO___9529260A2_I_>

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

CCTTGACGCA 10

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer OPE-6

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

AAGACCCCTC 10

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer OPE-12

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

TTATCGCCCC 10

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer OPE-19

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

BNSDOCID: <WO___9529260A2_I_>

ACCCCCGAAG 10

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer OPE-15

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

ACGCACAACC 10

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 627 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Pseudocercosporella herpotrichoides
(B) STRAIN: Strain W
(C) INDIVIDUAL ISOLATE: Variant W5-1

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..627
(D) OTHER INFORMATION: /note= "DNA sequence for the Internal Transcribed Spacer of Pseudocercosporella herpitrichoides strain W (variant W5-1)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

TCCGTAGGTG AACCTGCGGA AGGATCATTA ATAGAGCAAT GAACAGACAG CGCCCTGGGA 60

GAAATCCTGG GGGCTACCCT ACTTCGGTAG GGTTTAGAGT CGTCAGGCCT CTCGGAGAAG 120

CCTGGTTCAG ACCTCCACCC TTGAATAAAT TACCTTTGTT GCTTTGGCAG GGCGCCTCGC 180

GCCAGCGGCT TCGGCTGTTG AGTACCTGCC AGAGGACCAC AACTCTTGTT TTTAGTGATG 240

TCTGAGTACT ATATAATAGT TAAAACTTTC AACAACGGAT CTCTTGGTTC TGGCATCGAT 300

GAAGAACGCA GCGAAATGCG ATAAGTAATG TGAATTGCAG AATTCAGTGA ATCATCGAAT 360

CTTTGAACGC ACATTGCGCC CTCTGGTATT CCGGGGGGCA TGCCTGTTCG AGCGTCATTA 420

TAACCACTCA AGCTCTCGCT TGGTATTGGG GTTCGCGTCC TCGCGGCCTC TAAAATCAGT 480

BNSDOCID: <WO__9529260A2_I_>

GGCGGTGCCT CTCGGCTCTA CGCGTAGTAA TACTCCTCGC GATTGAGTCC GGTAGGTTTA 540

CTTGCCAGTA ACCCCCAATT TTTTACAGGT TGACCTCGGA TCAGGTAGGG ATACCCGCTG 600

AACTTAAGCA TATCAATAAG CGGAGGA 627

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: M13 universal -20 oligonucleotide primer

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

GTAAAACGAC GGCCAGT 17

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: M13 universal reverse oligonucleotide primer

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

AACAGCTATG ACCATG 16

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer JB563"

(iii) HYPOTHETICAL: NO

INSDOCID: <WO___9529260A2_I_>

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

CTTGCCTGCC GGTTGGACAA ATT 23

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide JB564"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

CTCAGTAGTT TACTACTGTA AAAGG 25

(2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide JB565"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

CTTCTGGACG CAAGTGTTTG TTAC 24

(2) INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide JB566"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

GTTTTTAGTG GAACTTCTGA GT 22

(2) INFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid

BNSDOCID: <WO___9529260A2_I_>

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide JB567"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

CGCAGGAACC CTAAACTCT 19

(2) INFORMATION FOR SEQ ID NO:55:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer JB568"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

GCCCGCCGCA GG 12

(2) INFORMATION FOR SEQ ID NO:56:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer JB569"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

RTWWTTWRTG GAMYYTCTGA GT 22

(2) INFORMATION FOR SEQ ID NO:57:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer JB570"

BNSDOCID: <WO__9529260A2_I_>

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

TATGTTGCCT CGGCGG 16

(2) INFORMATION FOR SEQ ID NO:58:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer JB571"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

TAACGATATG TAAATTACTA CGCT 24

(2) INFORMATION FOR SEQ ID NO:59:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer JB572"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

AAGTTGGGGT TTAACGGC 18

(2) INFORMATION FOR SEQ ID NO:60:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer JB573"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

BNSDOCID: <WO___9529260A2_I_>

AGCGAGCCCG CCAC 14

(2) INFORMATION FOR SEQ ID NO:61:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer JB574"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

CCATTGTGAA CGTTACCTAT AC 22

(2) INFORMATION FOR SEQ ID NO:62:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer JB575"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

CGACCAGAGC GAGATGTA 18

(2) INFORMATION FOR SEQ ID NO:63:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer JB576"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

GTGAACATAC CTTATGTTGC C 21

(2) INFORMATION FOR SEQ ID NO:64:

(i) SEQUENCE CHARACTERISTICS:

3NSDOCID: <WO___9529260A2_I_>

(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer JB577"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

GTTGCCTCGG CGGATC 16

(2) INFORMATION FOR SEQ ID NO:65:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer JB578"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

CCGCGACGAT TACCAG 16

(2) INFORMATION FOR SEQ ID NO:66:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer JB538'"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

TGACGACTCT AAACCCTACC A 21

(2) INFORMATION FOR SEQ ID NO:67:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

BNSDOCID: <WO___9529260A2_I_>

(A) DESCRIPTION: /desc = "Oligonucleotide primer JB539'"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

CGACGACTCT AAACCTTACC G 21

(2) INFORMATION FOR SEQ ID NO:68:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer W130"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

ATTCAAGGGT GGAGGTCTGA 20

(2) INFORMATION FOR SEQ ID NO:69:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer R130"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

ATTCAAGGGT GGAGGTCTGG 20

(2) INFORMATION FOR SEQ ID NO:70:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer JB539'15"

(iii) HYPOTHETICAL: NO

3NSDOCID: <WO___9529260A2_I_>

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

CTCTAAACCC TACCA 15

(2) INFORMATION FOR SEQ ID NO:71:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer JB539'15"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

CTCTAAACCT TACCG 15

(2) INFORMATION FOR SEQ ID NO:72:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer JB553"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:72:

GTGGTCCTCT GGCAG 15

(2) INFORMATION FOR SEQ ID NO:73:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer JB554"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:73:

CTCAACAGCC GAAGC 15

BNSDOCID: <WO___9529260A2_I_>

(2) INFORMATION FOR SEQ ID NO:74:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer JB555"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:74:

GGGTGGAGGT CTGA 14

(2) INFORMATION FOR SEQ ID NO:75:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer JB556"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:75:

GGTGGAGGTC TGG 13

(2) INFORMATION FOR SEQ ID NO:76:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer JB561"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:76:

TGGAGGTCTG GACCA 15

(2) INFORMATION FOR SEQ ID NO:77:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

3NSDOCID: <WO___9529260A2_I_>

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer JB562"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:77:

TGGAGGTCTG AACCA 15

(2) INFORMATION FOR SEQ ID NO:78:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer JB559"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:78:

AGGGTGGAGG TCTGA 15

(2) INFORMATION FOR SEQ ID NO:79:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer JB560"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:79:

AGGGTGGAGG TCTGG 15

(2) INFORMATION FOR SEQ ID NO:80:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer JB557"

BNSDOCID: <WO___9529260A2_I_>

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:80:

TTCTCCGAGA GGCCT 15

(2) INFORMATION FOR SEQ ID NO:81:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer JB558"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:81:

TTCTCCGAGA GGCCC 15

(2) INFORMATION FOR SEQ ID NO:82:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 504 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..504
(D) OTHER INFORMATION: /note= "DNA sequence for the internal transcribed spacer region of Fusarium culmorum (fculm.con)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:82:

GAGGGATCAT TACCGAGTTT ACTRACTCCC AAACCCCTGT GAACDTACCT TATGTTGCCT 60

CGGCGGATCA GCCCGCGCCC CGTAAAAAGG GACGGCCCGC CGCAGGAACC CTAAACTCTG 120

TTTTTAGTGG AACTTCTGAG TATAAAAAAC AAATAAATCA AAACTTTCAA CAACGGATCT 180

CTTGGTTCTG GCATCGATGA AGAACGCAGC AAAATGCGAT AAGTAATGTG AATTGCAGAA 240

TTCAGTGAAT CATCGAATCT TTGAACGCAC ATTGCGCCCG CCAGTATTCT GGCGGGCATG 300

CCTGTTCGAG CGTCATTTCA ACCCTCAAGC CCAGCTTGGT GTTGGGAGCT GCAGTCCTGC 360

TGCACTCCCC AAATACATTG GCGGTCACGT CGRAGCTTCC ATAGCGTAGT AATTTACATA 420

TCGTTACTGG TAATCGTCGC GGCYACGCCG TTAAACCCCA ACTTCTGAAT GTTGACCTCG 480

BNSDOCID: <WO___9529260A2_I_>

GATCAGGTAG GAATACCCGC TGAA 504

(2) INFORMATION FOR SEQ ID NO:83:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 503 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..503
(D) OTHER INFORMATION: /note= "DNA sequence for the internal transcribed spacer region of Fusarium graminearum (fgram.con)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:83:

GGATCATTAC CGAGTTTACW SACTCCCAAA CCCCTGTGAA CATACCTTAT GTTGCCTCGG 60

CGGATCAGCC CGCGCCCCGA AAGGGACGGC CCGCCGCAGG AACCCTAAAC TCTGTTTTTA 120

GTGGAACTTC TGAGTATAAA AAACAAATAA ATCAAAACTT TCAACAACGG ATCTCTTGGT 180

KCTGGCATCG ATGAAGAACG CASCRAAATG CGATAAGTAA TGTGWATTGC AGAATTCAGT 240

GAATCAWCGA ATCTTTGAAC GCWSATTGCK MCCRCCAGTA TTCTGGCGGG CATGCCTGTT 300

CGAGCGTCAT TTCAACCCTC AAGCCCAGVT TGGTGTKGGG GARYTGCAGK CCTRYTKCAC 360

TCCCCAAATA ARTTGGCGGT CACGTCGAAC TTCCATAGCG TAGTAAGTTA CACATCGTTA 420

CTGGTAATCG TCGCGGCTAC GCCGTTAAAC CCCAACTTCT GAATGTTGAC CTCGGATCAG 480

GTAGGAATAC CCGCTGAAGG TAA 503

(2) INFORMATION FOR SEQ ID NO:84:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 353 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..353
(D) OTHER INFORMATION: /note= "DNA sequence for the internal transcribed spacer region of Fusarium moniliforme (fmono.con)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:84:

TCCGTAGGTG AACCTGCGGA TAGGRGTCAT TASMGAGTTT ACWACTSCCA AACCCCTGTG 60

AAYATACCTT ATGTTGCSTC GGCGGATCAG CCCGCGCSCC GTARRAAGGG ACGGCCCGCC 120

BNSDOCID: <WO__9529260A2_I_>

GCAGGAACCC TAAACTCTGT TTTTAGTGGA ACTTCTGAGT ATAAAAAACA AATAAATCAA 180

AACTTTCAAC AACGGATCTC TTGGTTCTGG CATCGATGAA GAACGCAGCA AAATGCGATA 240

AGTAATGTGA ATTGCAGAAT TCAGTGAATC ATCGAATCTT TGAACGCACA TTGYGMCCGC 300

CAGTATTCTG GCGGGCATGC CTGTTCGAGC GTCATTTCAA CCCTCAAGCC CAG 353

(2) INFORMATION FOR SEQ ID NO:85:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 545 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..545
(D) OTHER INFORMATION: /note= "DNA sequence for the internal transcribed spacer region of Microdochium nivale (mnivale.txt)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:85:

GCGGATCATT ACAGAGTTGC AAAACTCCCT AAACCATTGT GAACGTTACC TATACCGTTG 60

CTTCGGCGGG CGGCCCCGGG GTTTACCCCC CGGRAGYCCC TGGKMCCCAC CGCGGGSGCC 120

MGCCGGAGGT CACCAAACTC TTGATAATTT ATGGCCTCTC TGAGTCTTCT GTACTGAATA 180

AGTCAAAACT TTCAACAACG GATCTCTTGG TTCTGGCATC GATGAAGAAC GCAGCGAAAT 240

GCGATAAGTA ATGTGAATTG CAGAATTCAG TGAATCATCG AATCTTTGAA CGCACATTGC 300

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CGCACCGAGM GTAGTAGSAT ACATCTCGCT CTGGTCGCGC CGCGGGTTCC GGCCGTTAAA 480

CCACCTTTTT AACCCAAGGT TGACCTCGGA TCAGGTAGGA AGACCCGCTG AACTTACGCA 540

TATCA 545

(2) INFORMATION FOR SEQ ID NO:86:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 563 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..563
(D) OTHER INFORMATION: /note= "DNA sequence for the

BNSDOCID: <WO___9529260A2_I_>

internal transcribed spacer of Septoria avenae f. sp. tricicea ATCC# 26380 (satits.con)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:86:

TCCCGTAGGT GAACCTGCGG AAGGATCATT ACACTCAGTA GTTTACTACT GTAAAGGAGG 60

CTGTTAGTCT GTATAGCGCA AGCTGATGAG CAGCTAGCCT CTTTTATCCA CCCTTGTCTT 120

TTGCGTACCC ACGTTTCCTC GGCAGGCTTG CCTGCCGATT GGACAAACCT ATAACCTTTT 180

TAATTTTCAA TCAGCGTCTG AAAAACTTAA TAATTACAAC TTTCAACAAC GGATCTCTTG 240

GTTCTGGCAT CGATGAAGAA CGCAGCGAAA TGCGATAAGT AGTGTGAATT GCAGAATTCA 300

GTGAATCATC GAATCTTTGA ACGCACATTG CGCCCCTTGG TATTCCATGG GGCATGCCTG 360

TTCGAGCGTC ATTTGTACCC TCAAGCTCTG CTTGGTGTTG GGTGTTTGTC CTCTCCCTAG 420

TGTTTGGACT CGCCTTAAAA TAATTGGCAG CCAGTGTTTT GGTAYTGAAG CGCAGCACAA 480

GTCGCGATTC TTATCAAATA CTTGCGTCCA CAAGCCCTTT TTTAACTTTT GACCTCGGAT 540

CAGGTAGGAG ACCGCTGACT TAA 563

BNSDOCID: <WO___9529260A2_I_>

What is claimed is:

1. A DNA sequence encoding an Intervening Transcribed Sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO:2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 47, SEQ ID NO: 82, SEQ ID NO: 83, and SEQ ID NO: 84, SEQ ID NO: 85, and SEQ ID NO: 86.

2. An oligonucleotide primer for use in amplification-based detection of a fungal Intervening Transcribed Sequence wherein said primer is derived from the DNA sequence of claim 1.

3. The oligonucleotide of claim 2, wherein said primer is selected from the group consisting of SEQ ID NOS: 7 to 37 and SEQ ID NOS: 50 to 65.

4. A pair of oligonucleotide primers for use in the amplification-based detection of a fungal Intervening Transcribed Sequence, wherein at least one primer is selected from the group consisting of SEQ ID NOS: 7 to 37 and SEQ ID NOS: 50 to 65.

5. The pair of oligonucleotide primers according to claim 4, wherein one primer is selected from the group consisting of SEQ ID NOS: 7 to 37 and SEQ ID NOS: 50 to 65 and the other primer is selected from the group consisting of SEQ ID NOS: 38 to 41.

6. The pair of oligonucleotide primers according to claim 4, wherein said pair is selected from the group consisting of pairs of Table 4.

7. The pair of oligonucleotide primers according to claim 4 wherein said pair is selected from the group consisting SEQ ID NO: 7 and SEQ ID NO: 8.

8. The pair of oligonucleotide primers according to claim 5, wherein said pair is selected from the group consisting of

(a) SEQ ID NO: 10 and SEQ ID NO: 9;

(b) SEQ ID NO: 12 and SEQ ID NO: 38;

BNSDOCID: <WO___9529260A2_I_>

(c) SEQ ID NO: 11 and SEQ ID NO: 41;

(d) SEQ ID NO: 29 and SEQ ID NO: 38;

(e) SEQ ID NO: 7 and SEQ ID NO: 41;

(f) SEQ ID NO: 30 and SEQ ID NO: 38;

(g) SEQ ID NO: 15 and SEQ ID NO: 19;

(h) SEQ ID NO: 17 and SEQ ID NO: 22;

(i) SEQ ID NO: 18 and SEQ ID NO: 20; and

(j) SEQ ID NO: 26 and SEQ ID NO: 41.

9. An oligonucleotide primer for identification of a fungal pathogen, wherein said primer is selected from the group of primers consisting of SEQ ID NO: 42 to 46.

10. A method for the detection of a fungal pathogen, comprising the steps of:

(a) isolating DNA from a plant leaf infected with a pathogen;

(b) amplifying a part of the intervening transcribed region of said pathogen using said DNA as a template in a polymerase chain reaction with a pair of primers according to claims 4 or 5; and

(c) visualizing said amplified part of the intervening transcribed region.

11. The method of claim 10, wherein said fungal pathogen is selected from *S. nodorum, S. tritici, P. herpotrichoides, M. fijiensis, M. musicola, F. culmorum, F. graminearum, Microdochium. nivale,* and *F. moniliforme.*

12. The method of claim 10, wherein said *P. herpotrichoides* is selected from strain W and strain R.

13. A method for the detection of a fungal pathogen, comprising the steps of:

(a) isolating DNA from a plant leaf infected with a pathogen;

(b) subjecting said DNA to polymerase chain reaction amplification using at least one primer according to claim 9; and

(c) visualizing the product or products of said polymerase chain reaction amplification.

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14. A kit comprising a carrier being compartmentalized to receive in close confinement therein one or more container means, one of said container means containing the primer of claim 2.

15. A kit comprising a carrier being compartmentalized to receive in close confinement therein one or more container means, one of said container means containing the primer of claim 3.

16. A kit comprising a carrier being compartmentalized to receive in close confinement therein one or more container means, one of said container means containing the primers of claim 4.

17. A kit comprising a carrier being compartmentalized to receive in close confinement therein one or more container means, one of said container means containing the primer of claim 5.

18. A kit comprising a carrier being compartmentalized to receive in close confinement therein one or more container means, one of said container means containing the primer of claim 13.

19. In a quantitative colorimetric assay for the detection of a fungal pathogen comprising the steps of (a) isolating DNA from a plant leaf infected with a pathogen; (b) amplifying the DNA region of said pathogen in a polymerase chain reaction; and (c) visualizing said amplified part of the intervening transcribed region

wherein said improvement comprises amplifying said DNA from a part of the intervening transcribed region of said pathogen using as a template a pair of primers according to claims 4 as the diagnostic primers and visualizing said amplified part using a capture primer wherein said capture primer is selected from the group consisting of a primer of Table 5.

20. The assay of claim 19 wherein said diagnostic primers are selected from SEQ ID NO.: 10 and SEQ ID NO.: 9 and the capture primer is SEQ ID NO.: 39.

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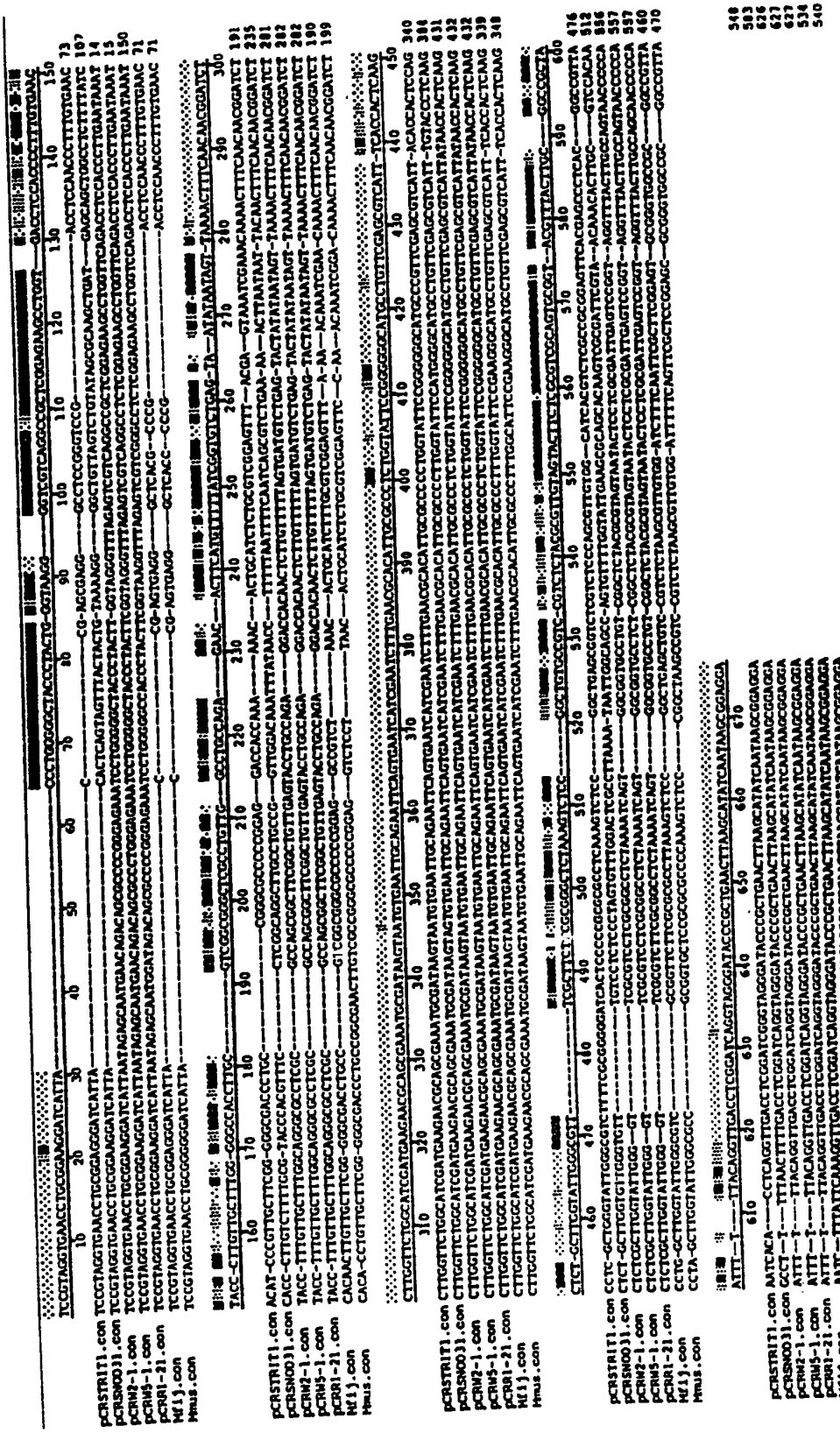
21. The assay of claim 19 wherein said diagnostic primers are selected from SEQ ID NO.: 18 and SEQ ID NO.: 20 and the capture primer is SEQ ID NO.: 71.

22. The assay of claim 19 wherein said diagnostic primers are selected from SEQ ID NO.: 15 and SEQ ID NO.: 19 and the capture primer is SEQ ID NO.: 71.

BNSDOCID: <WO___9529260A2_I_>

FIGURE 1



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BNSDOCID: <WO__9529260A2_I_>

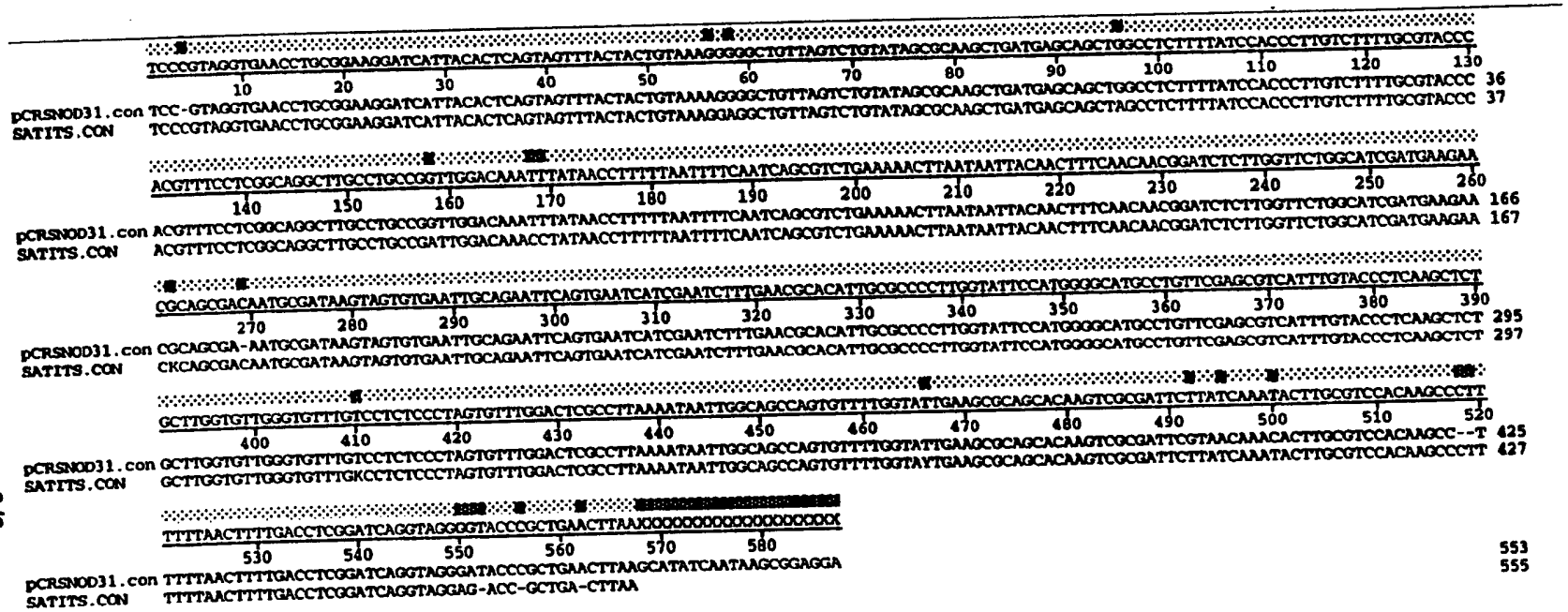

FIGURE 2

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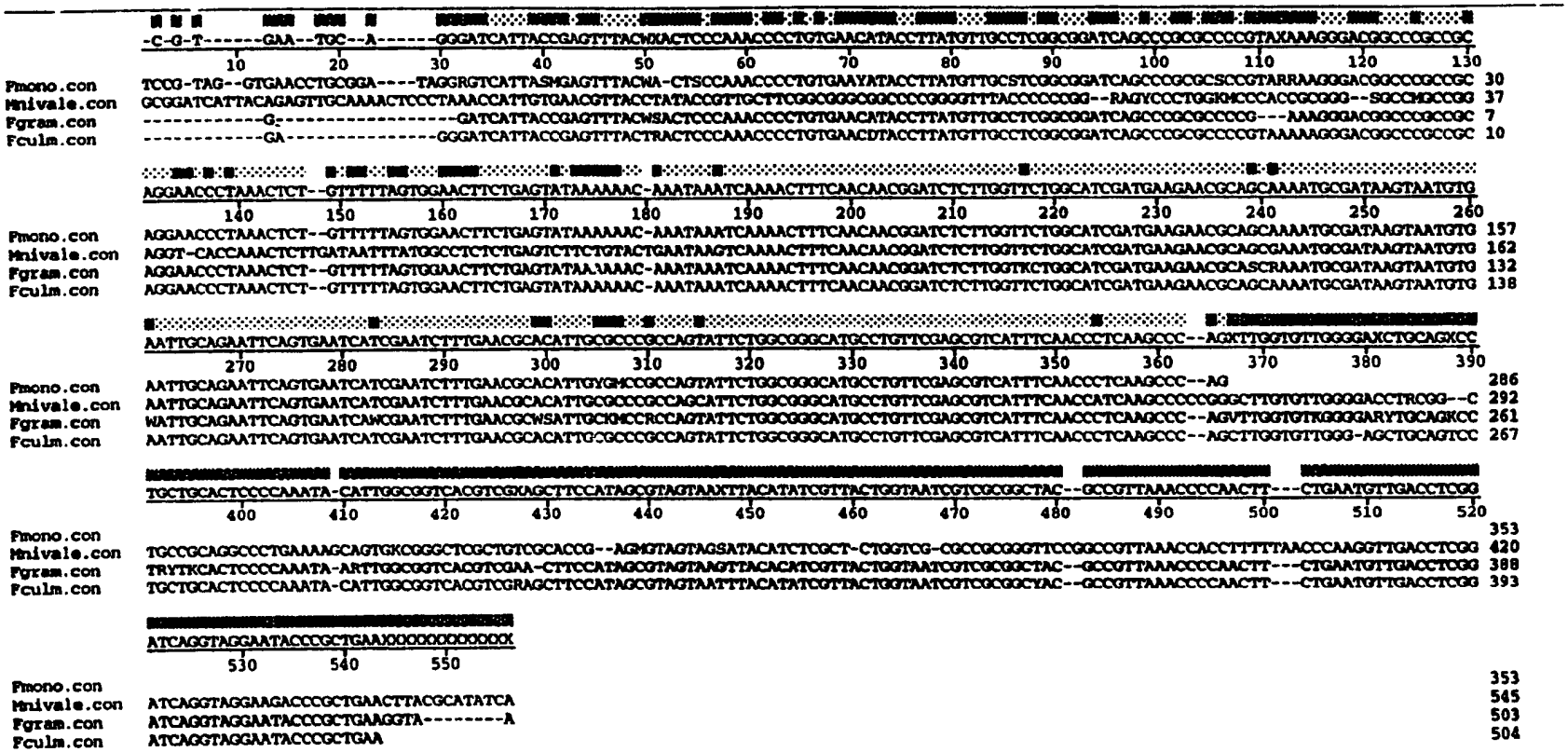

FIGURE 3

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(74) Agent: WALSH, Andrea, C.; 7 Skyline Drive, Hawthorne, NY 10532 (US).

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(57) Abstract

DNA sequences from the Internal Transcribed Spacer of the ribosomal RNA gene region are described for different species and strains of *Septoria*, *Pseudocercosporella*, *Fusarium* and *Mycosphaerella*. Specific primers from within these sequences are identified as being useful for the identification of the fungal isolates using PCR-based techniques.

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INTERNATIONAL SEARCH REPORT

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PCT/US 95/04712

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C07H21/04 C12P19/34

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PHYSIOLOGICAL AND MOLECULAR PLANT PATHOLOGY, vol.41, pages 179 - 88 XUE, B. ET AL. 'pathotype identification of Leptosphearia maculans with PCR and oligonucleotide primers from ribosomal internal transcribed spacer sequences' see the whole document ---	1,2,10, 11,14-18
X	PHYTOPATHOLOGY, vol.84, no.5, pages 478- - 42 TISSERAT, N. ET AL 'selective amplification of rDNA internal spacer regions to detect Ophiosphaerella korrae and O. herptotricha' see the whole document ---	1,2,10, 11,14-18

-/--

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[X] Patent family members are listed in annex.

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Microtitration Plate Enzyme Immunoassay To Detect PCR-Amplified DNA from *Candida* Species in Blood

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We developed a microtitration plate enzyme immunoassay to detect PCR-amplified DNA from *Candida* species. Nucleotide sequences derived from the internal transcribed spacer (ITS) region of fungal rDNA were used to develop species-specific oligonucleotide probes for *Candida albicans*, *C. tropicalis*, *C. parapsilosis*, and *C. krusei*. No cross-hybridization was detected with any other fungal, bacterial, or human DNAs tested. In contrast, a *C. (Torulopsis) glabrata* probe cross-reacted with *Saccharomyces cerevisiae* DNA but with no other DNAs tested. Genomic DNA purified from *C. albicans* blastoconidia suspended in blood was amplified by PCR with fungus-specific universal primers ITS3 and ITS4. With the *C. albicans*-specific probe labeled with digoxigenin, a biotinylated capture probe, and streptavidin-coated microtitration plates, amplified DNA from as few as two *C. albicans* cells per 0.2 ml of blood could be detected by enzyme immunoassay.

Disseminated candidiasis is an important infectious complication in patients who have undergone cardiac or abdominal surgery or in patients who are severely granulocytopenic as a result of therapies for bone marrow transplantation or cancer (16, 27, 29). Antemortem diagnosis of disseminated candidiasis is difficult because the clinical presentation is usually nonspecific and antibody production in immunocompromised patients can be variable, complicating the diagnosis (14). Although two or more positive blood cultures are often used to identify disseminated disease, standard blood culturing methods can require 2 to 5 days for detection and even longer for species identification (14). To shorten the time required to obtain an accurate diagnosis independent of a functioning immune system, laboratory tests have been developed to detect circulating *Candida* cell wall mannan (8, 21), enolase (35), or D-arabinitol (8, 14) for rapid diagnosis of disseminated candidiasis. However, the sensitivity of these tests varies among investigators and is reported to range from 22 to 100% (14).

The development of DNA-based methods for detection of *Candida* spp. provides an alternative and potentially more sensitive means to diagnose disseminated candidiasis. Southern blotting of nonamplified DNA targets has a detection limit of approximately 500 to 10^5 blastoconidia, depending on the method and probe used (6, 11). PCR technology (30) was recently adapted to amplify *Candida albicans* DNA, facilitating its detection (3, 7, 10, 25). However, detection of *C. albicans* DNA recovered from clinical specimens, even after PCR amplification, lacks sensitivity and is cumbersome for most laboratories (3, 7), particularly when DNA is recovered from blood (10, 24). Sensitivity can be improved to 10 cells per ml (15) or 3 cells per 0.1 ml (24), but this requires the use of Southern blotting coupled with radioisotopically labeled probes for detection.

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In the present study, *Candida* sp. 5.8S rRNA genes and the adjacent internal transcribed spacer (ITS) regions were PCR amplified by using fungus-specific universal primers ITS3 and ITS4 (36). Nonisotopic, digoxigenin-labeled oligonucleotide probes were designed on the basis of the sequence of the ITS2 region of *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, and *C. (Torulopsis) glabrata* rDNA (17). These probes were then used in a microtitration plate enzyme immunoassay (EIA) to rapidly detect and identify amplified genomic DNA from *C. albicans* blastoconidia introduced into blood.

MATERIALS AND METHODS

Microorganisms and reagents. *C. albicans* B311 and H317 and *Saccharomyces cerevisiae* AB972 were obtained as previously described (19). *C. tropicalis* WO745, *C. parapsilosis* WO471, *C. guilliermondii* WO411, *C. krusei* WO701, *C. (Torulopsis) glabrata* WO756, and *Cryptococcus neoformans* var. *neoformans* 90-6 were obtained from lyophilized stock cultures maintained in the Mycology Culture Collection, Emerging Bacterial and Mycotic Diseases Branch, Centers for Disease Control and Prevention (CDC). The identity of yeast isolates was determined by carbohydrate assimilation tests performed with the API20C kit (bioMerieux Vitek, Inc., Hazelwood, Mo.), germ tube formation in serum, morphology on cornmeal agar, or for *C. neoformans* identification, pigment production on DL-3,4-dihydroxyphenylalanine agar. Filamentous fungal isolates *Aspergillus fumigatus* 91-019720, *A. flavus* 91-019724, *Histoplasma capsulatum* G217B, *Penicillium marneffei* B-3420, and *Blastomyces dermatitidis* 4478 were obtained from the Mycology Culture Collection, Emerging Bacterial and Mycotic Diseases Branch, CDC. Filamentous fungi were identified by colonial and microscopic morphology. *Escherichia coli* DH5α was obtained from Bethesda Research Laboratories (Gaithersburg, Md.). *Staphylococcus aureus* ATCC 1126 DNA was kindly provided by Tammy Bannerman (Hospital Infections Program, CDC), and *Pseudomonas aeruginosa* ATCC 10332 DNA was kindly provided by Arnold Steigerwalt (Division of Bacterial and Mycotic Diseases, CDC). Genomic DNA from human placenta was kindly provided by David Swan (Division of Viral and Rickettsial Diseases, CDC). All chemicals were purchased from Sigma Chemical Co., St. Louis, Mo., unless otherwise specified.

Precautions against contamination. Universal precautions suggested by Kwok and Higuchi (18) were used to eliminate possible contamination of samples. Cross-contamination by aerosols was reduced by physical separation of laboratory areas used to prepare PCRs and to analyze PCR products and by using a combination of positive-displacement pipetters and aerosol-resistant pipette tips. Other precautions included autoclaving of buffers and distilled water used for PCRs, use of fresh lots of previously aliquoted PCR reagents, and testing of appropriate negative controls, including omission of either the primer or the DNA template during the PCR.

Purification of target DNA. *Candida* species and *S. cerevisiae* isolates were grown in 10 ml of YPG broth (1% yeast extract, 2% Bacto Peptone, 1% glucose; Difco Laboratories, Detroit, Mich.) at 37°C, and DNA was purified from lysed spheroplasts as described by Lasker et al. (20). *C. neoformans* genomic DNA was purified from lysed spheroplasts as described by Restrepo and Barbour (28).

Filamentous fungi were grown in 50 ml of YPG broth at 30°C for 48 h. Mycelia were harvested by filtration, washed once with sterile distilled water, and ground with a mortar and pestle in the presence of liquid nitrogen in a laminar flow biological safety cabinet. Genomic DNA was then isolated as described by Spitzer et al. (33) by repeated phenol-chloroform and chloroform extractions. Bacterial DNA was isolated by standard methods (23).

Preparation of template DNA from *C. albicans* blastoconidia suspended in blood. Blood (10 ml) from New Zealand White rabbits (Myrtle's Rabbitry, Memphis, Tenn.) was collected from a central ear artery into lysis-centrifugation tubes (Wampole Laboratories, Cranbury, N.J.) in accordance with CDC Animal Care and Use Committee guidelines. Human blood was also tested in preliminary experiments with similar results.

To determine the sensitivity of the prototype test, blood was seeded with known numbers of *Candida* blastoconidia. Knowing the exact number of (viable plus nonviable) cells present was important for determining test sensitivity, since PCR methods can detect DNA from dead, as well as viable, blastoconidia (unpublished observation). Therefore, 0, 10^1, 10^2, 10^3, or 10^4 *C. albicans* B311 blastoconidia which had been enumerated microscopically with a hemacytometer were seeded into 1 ml of collected blood. Erythrocytes and leukocytes were lysed by adding 0.8 ml of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) containing 0.05% (wt/vol) proteinase K (15 U/mg) and 0.05% (vol/vol) Tween 20 detergent to 0.2 ml of seeded blood. After incubation at 55°C for 30 min, blastoconidia were centrifuged at 10,000 × *g* for 8 min at 20°C in a Beckman Microfuge and washed twice with TE buffer containing 0.5% Tween 20 and twice with SE solution (1 M sorbitol–0.1 M EDTA, pH 7.5).

Spheroplasts were prepared by adding 0.5 ml of SE solution containing 0.1% (wt/vol) Zymolyase-100T (100,000 U/g; Seikagaku Corp., Tokyo, Japan) and 1% (vol/vol) 2-mercaptoethanol to the cell pellets. After incubation at 37°C for 30 min and an additional 30 min of incubation at 25°C on a rocker platform (20 cycles per min), spheroplasts were centrifuged at 7,000 × *g* for 5 min at 20°C and washed twice with SE solution. Spheroplasts were lysed in 0.4 ml of TE buffer containing 0.05% (wt/vol) proteinase K (15 U/mg) and 0.5% (vol/vol) Tween 20, incubated at 55°C for 1 h, and then heated at 95°C for 10 min to inactivate the proteinase K. Nucleic acids were extracted by adding an equal volume of TE-saturated phenol-chloroform (1 volume of TE-saturated phenol to 1 volume of chloroform) and vortex mixing for 1 min. The emulsion was centrifuged at 10,000 × *g* for 5 min, and the aqueous phase was extracted by adding an equal volume of chloroform. One-tenth volume of 3.0 M sodium acetate buffer (pH 5.2) was then added to the resultant aqueous phase, and DNA was precipitated by adding 2 volumes of cold isopropanol and placing the samples at −20°C for 1 h. Precipitated nucleic acids were then collected by centrifugation at 10,000 × *g* for 10 min at 4°C. One milliliter of 70% ice-cold ethanol was added to wash the pellet, and samples were centrifuged for an additional 5 min at 10,000 × *g*. Nucleic acids were then vacuum dried and resuspended in 30 μl of distilled water or TE buffer.

Oligonucleotide synthesis of primers and probes. Synthetic oligodeoxyribonucleotides were prepared by β-cyanoethyl phosphoramidite chemistry with a 380B automated DNA synthesizer (Applied Biosystems, Foster City, Calif.). The oligonucleotide primer pair ITS3 and ITS4 was previously shown to amplify fungal 5.8S rDNA and the adjacent ITS region (36). Oligonucleotide probes specific for *Candida* species were prepared from sequences of the rDNA ITS2 region (17, 22, 36). Oligonucleotide probes CA, CT, CP, CK, and CG were designed to detect *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, and *C. (Torulopsis) glabrata*, respectively. For probes CA, CT, and CP, nonhomologous 3′ regions of ITS2 were derived from GenBank entries L07796, L11349, and L11352, respectively. Probes CG and CK were developed on the basis of data previously reported (17).

Oligonucleotide probes were initially synthesized with a 5′-terminal amine group (Aminolink 2; ABI, Foster City, Calif.). Amino-linked oligonucleotides were mixed with a 10-fold molar excess of digoxigenin-3-*O*-methylcarbonyl-ε-aminocaproic acid *N*-hydroxysuccinimide ester (Boehringer Mannheim, Indianapolis, Ind.) in 0.1 M sodium carbonate buffer, pH 9.0. After overnight incubation at ambient temperature, the digoxigenin-conjugated oligonucleotides were purified by reverse-phase high-pressure liquid chromatography (2).

The 5.8S rDNA consensus oligonucleotide probe BP was labeled with biotin by incorporating dimethoxytrityl-biotin-carbon 6-phosphoramidite (Cambridge Research Biochemicals, Inc., Wilmington, Del.) at the 5′ end during standard synthesis on a 380B DNA synthesizer. The dimethoxytrityl group was retained on the biotinylated oligonucleotide to facilitate purification by reverse-phase high-pressure liquid chromatography (2). ITS4P (5′-end-protected 3′ primer) was synthesized as previously described (34).

PCR conditions. A Gene Amp DNA amplification reagent kit (Perkin-Elmer Corp., Norwalk, Conn.) was used for PCR amplification of genomic DNA. The reaction mixture (50 μl) contained 50 mM KCl; 1.5 mM $MgCl_2$; 0.001% gelatin; 10 mM Tris-HCl (pH 8.3); 50 μM each dATP, dCTP, dGTP, and dTTP; 0.1 μM each primer; 5 μl of template DNA, and 1.25 U of *Taq* DNA polymerase. A primer concentration of 0.1 μM was found by titration to be optimal for DNA amplification with minimal primer dimerization (unpublished observations). Samples were overlaid with 30 μl of mineral oil prior to PCR amplification in a Perkin Elmer Cetus DNA thermal cycler. *Taq* polymerase was added after the thermal cycler reached 94°C and before initiation of temperature cycling. PCR amplification was determined to be optimum after 30 cycles of denaturation at 95°C for 1 min, primer annealing at 55°C for 1 min, and extension at 72°C for 1.5 min. After the last cycle, final DNA extension was performed at 72°C for 5 min.

Agarose gel electrophoresis. Electrophoresis was conducted in TBE buffer (0.089 M Tris, 0.089 M boric acid, 0.02 M EDTA, pH 8.4) at 80 V for 1 to 2 h on gels composed of 1% (wt/vol) agarose (International Technologies, Inc. New Haven, Conn.) and 1% (wt/vol) NuSieve (FMC Bioproducts, Rockland, Maine).

Microtitration plate hybridization assay. PCR-amplified DNA was hybridized to digoxigenin- and biotin-labeled oligonucleotide probes and detected in an EIA by capture with streptavidin-coated microtitration plates (Immulon 2; Dynatech Laboratories, Inc., Chantilly, Va.) (9). To facilitate hybridization, single-stranded DNA was prepared from double-stranded PCR products by either exonuclease digestion of the non-phosphothioate-protected strand (9) or heat denaturation as described below.

Ten microliters of the PCR product was supplemented with dithiothreitol to 1 mM and digested with 0.4 U of T7 gene 6 exonuclease (United States Biochemical, Cleveland, Ohio) per μl for 15 min at 37°C. The digested product was then heated at 75°C for 15 min to inactivate the exonuclease. Alternatively, 10 μl of the PCR product was heated at 95°C for 5 min and then immediately cooled on ice. The single-stranded PCR product obtained by either exonuclease digestion or heating was added to 0.2 ml of hybridization solution (4× SSC [saline sodium citrate buffer; 0.6 M NaCl, 0.06 M trisodium citrate, pH 7.0] containing 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES], 2 mM EDTA, and 0.15% [vol/vol] Tween 20) supplemented with 50 ng each of biotin- and digoxigenin-labeled probes per ml.

Hybridization reactions were performed in 0.5-ml Eppendorf tubes at 37°C for 1 h. After hybridization, 100 μl of each sample was added to duplicate wells of the streptavidin-coated microtitration plate (9), and the plate was incubated at ambient temperature for 1 h with shaking (Minishaker, manufactured for Dynatech by CLTI, Middletown, N.Y.). After washing with potassium phosphate-buffered saline containing 0.05% Tween 20, 100 μl of peroxidase-conjugated, anti-digoxigenin Fab fragment (Boehringer Mannheim) diluted 1:2,000 in hybridization buffer was added per well. Plates were subsequently washed six times with potassium phosphate-buffered saline–0.05% Tween 20. Each well received 100 μl of a mixture of 1 volume of 3, 3′, 5, 5′-tetramethylbenzidine peroxidase substrate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) and 1 volume of peroxidase solution (Kirkegaard & Perry). The plates were then placed at ambient temperature for 15 min, and the A_{650} of each well was determined with a microtitration plate reader (UV Max; Molecular Devices, Inc., Menlo Park, Calif.). The absorbance of a reagent blank, in which the test sample was replaced with distilled water, was subtracted from each test sample.

Statistical analyses. Student's *t* test was used to determine significant differences between means plus or minus the standard deviation from the mean. *P* values of <0.05 were considered significant.

RESULTS

PCR amplification of fungal rDNA with universal fungal primers ITS3 and ITS4. All of the oligodeoxyribonucleotide primers and oligonucleotide probes used in this study are described in Table 1. One nanogram of genomic DNA from each isolate was amplified by PCR with the universal fungal primer pair ITS3 and ITS4. Use of this primer pair resulted in amplification of DNAs from all of the fungi examined, including *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. guilliermondii*, *C. krusei*, *C. (Torulopsis) glabrata*, *S. cerevisiae*, *C. neoformans*, *A. fumigatus*, *A. flavus*, *P. marneffei*, *B. dermatitidis*, and *H. capsulatum* (Fig. 1). No amplicon was detected with DNA isolated from *S. aureus*, *E. coli*, *P. aeruginosa*, or a human placental cell line. The following amplicon sizes were obtained for *Candida* species: 330 bp for *C. albicans*, 325 bp for *C. tropicalis*, 310 bp for *C. parapsilosis*, 260 bp for *C. guilliermondii*, 335 bp for *C. krusei*, and 410 bp for *C. (Torulopsis) glabrata* (Fig. 1). Fungi tested not belonging to a *Candida* species yielded amplicons with sizes ranging from 340 to 410 bp (Fig. 1).

Comparison of EIA sensitivity for detection of a PCR product by heat denaturation versus exonuclease digestion. Two methods for the production of single-stranded capture DNA to coat microtitration plates were compared. One method used heat to denature DNA into single strands, whereas the other